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| (54) Title: BIOCONJUGATION OF MACROMOLECULES | | | |
| (57) Abstract <p>This invention discloses a novel method for conjugating macromolecules to other molecular entities. Specifically, this invention discloses a method for conjugating or derivatizing macromolecules, such as oligonucleotides and proteins, using cycloaddition reactions, such as the Diels-Alder reaction or 1,3-dipolar cycloadditions. Included in the invention are the novel bioconjugated macromolecules that can be prepared according to the method of the invention.</p> | | | |

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BIOCONJUGATION OF MACROMOLECULES

FIELD OF THE INVENTION

This invention describes a novel method for conjugating macromolecules to other molecular entities. Particularly, this invention describes a method for conjugating or derivatizing oligonucleotides and proteins using cycloaddition reactions, such as the Diels-Alder reaction or 1,3-dipolar cycloaddition reactions.

BACKGROUND OF THE INVENTION

A method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules has been developed. This method, Systematic Evolution of Ligands by Exponential Enrichment, termed SELEX, is described in United States Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by Exponential Enrichment," now abandoned; United States Patent Application Serial No. 07/714,131, filed June 10, 1991, entitled "Nucleic Acid Ligands," now United States Patent No. 5,475,096; United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled "Methods for Identifying Nucleic Acid Ligands," now United States Patent No. 5,270,163 (see also WO 91/19813), each of which is specifically incorporated by reference herein. Each of these applications, collectively referred to herein as the SELEX Patent Applications, describes a fundamentally novel method for making a nucleic acid ligand to any desired target molecule. The SELEX process provides a class of products which are referred to as nucleic acid ligands (also referred to in the art as "aptamers"), each ligand having a unique sequence and property of binding specifically to a desired target compound or molecule.

The SELEX method involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of nucleic acids, preferably comprising a segment of randomized sequence, the SELEX method includes steps of contacting the mixture with the target under conditions favorable for binding, partitioning unbound nucleic acids from those nucleic acids which have bound

specifically to target molecules, dissociating the nucleic acid-target complexes, amplifying the nucleic acids dissociated from the nucleic acid-target complexes to yield a ligand-enriched mixture of nucleic acids, then reiterating the steps of binding, partitioning, dissociating and amplifying through as many cycles as desired to yield highly specific high affinity nucleic acid ligands to the target molecule.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed October 14, 1992, entitled "Method for Selecting Nucleic Acids on the Basis of Structure," abandoned in favor of United States Patent Application Serial No. 08/198,670, describes the use of SELEX in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA. United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," describes a SELEX based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or photocrosslinking and/or photoinactivating a target molecule. United States Patent Application Serial No. 08/134,028, filed October 7, 1993, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," abandoned in favor of United States Patent Application Serial No. 08/443,957, now United States Patent No. 5,580,737, describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, which can be non-peptidic, termed Counter-SELEX. United States Patent Application Serial No. 08/143,564, filed October 25, 1993, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Solution SELEX," abandoned in favor of United States Patent Application Serial No. 08/461,069, now United States Patent No. 5,567,588, describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule.

The SELEX method encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or

phosphate and/or base positions. SELEX-identified nucleic acid ligands containing modified nucleotides are described in United States Patent Application Serial No. 08/117,991, filed September 8, 1993, entitled "High Affinity Nucleic Acid Ligands containing Modified Nucleotides," abandoned in favor of United States Patent Application Serial No. 08/430,709, now United States Patent No. 5,660,985, that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. United States Patent Application Serial No. 09/134,028, *supra*, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH₂), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2'-Modified Nucleosides by Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent Application Serial No. 08/284,063, filed August 2, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," now United States Patent No. 5,637,459 and United States Patent Application Serial No. 08/234,997, filed April 28, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," now United States Patent No. 5,683,867, respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

The SELEX method encompasses complexes of oligonucleotides. United States Patent Application Serial No. 08/434,465, filed May 4, 1995 entitled "Nucleic Acid Ligand Complexes," describes a method for preparing a therapeutic or diagnostic complex comprised of a nucleic acid ligand and a lipophilic compound or a non-immunogenic, high molecular weight compound.

Nucleic acid ligands derived by the SELEX process have been used in diagnostic applications. (See e.g., United States Patent Application No. 08/487,425,

filed June 7, 1995, entitled "Enzyme Linked Oligonucleotide Assays (ELONAS)," United States Patent Application No. 08/479,729, filed June 7, 1995, entitled "Use of Nucleic Acid Ligands in Flow Cytometry," and United States Patent Application No. 08/628,356, filed April 5, 1996, entitled "Method for Detecting a Target Compound
5 in a Substance Using a Nucleic Acid Ligand." The full text of the above described patent applications, including but not limited to, all definitions and descriptions of the SELEX process, are specifically incorporated by reference herein in their entirety.

Considerable research is being directed to the application of oligonucleotides and oligonucleotide analogs as diagnostic and research reagents and as potential
10 therapeutic agents. There are currently at least three areas of exploration regarding the use of oligonucleotides as pharmaceutical compounds. In the most advanced field, antisense oligonucleotides are used to bind to certain coding regions in an organism to prevent the expression of proteins or to block various cell functions. Additionally, the discovery of RNA species with catalytic functions -- ribozymes --
15 has led to the study of RNA species that serve to perform intracellular reactions that will achieve desired effects. And lastly, the discovery of the SELEX process (Systematic Evolution of Ligands by Exponential Enrichment) (Tuerk and Gold (1990) *Science* 249:505) has shown that oligonucleotides can be identified that will bind to almost any biologically interesting target.

20 The use of antisense oligonucleotides as a means for controlling gene expression and the potential for using oligonucleotides as possible pharmaceutical agents has prompted investigations into the introduction of a number of chemical modifications into oligonucleotides to increase their therapeutic activity and stability. Such modifications are designed to increase cell penetration of the oligonucleotides,
25 to stabilize them from nucleases and other enzymes that degrade or interfere with the structure or activity of the oligonucleotide analogs in the body, to enhance their binding to targeted RNA, to provide a mode of disruption (terminating event) once sequence-specifically bound to targeted RNA and to improve the pharmacokinetic properties of the oligonucleotides.

30 Recent research has shown that RNA secondary and tertiary structures can have important biological functions (Tinoco *et al.* (1987) Cold Spring Harb. Symp.

Quant. Biol. 52:135; Larson *et al.* (1987) Mol. Cell. Biochem. 74:5; Tuerk *et al.* (1988) Proc. Natl. Acad. Sci. USA 85:1364; Resnekov *et al.* (1989) J. Biol. Chem. 264:9953). PCT Patent Application Publication WO 91/14436, entitled "Reagents and Methods for Modulating Gene Expression Through RNA Mimicry," describes
5 oligonucleotides or oligonucleotide analogs which mimic a portion of RNA able to interact with one or more proteins. The oligonucleotides contain modified internucleoside linkages rendering them nuclease-resistant, have enhanced ability to penetrate cells, and are capable of binding target oligonucleotide sequences.

The use of oligonucleotides as therapeutic and diagnostic agents is growing
10 rapidly with many compounds in human clinical trials. In many of these applications the oligonucleotide is derivatized or conjugated with another molecular entity. These conjugations are typically performed for the purpose of attaching fluorescent dyes or other diagnostic reporter groups or for attaching compounds that modulate the activity or the pharmacokinetic behavior of the oligonucleotide. For example, Smith
15 *et al.* describe the synthesis of fluorescent dye-conjugated primers for use in fluorescence-based DNA sequence analysis (Smith *et al.* (1987) Methods Enzymol. 155: 260-301). United States Patent No. 5,650,275 of Pitner *et al.*, describes the use of spectroscopically detectable labeled nucleic acid ligands to determine the presence or absence of a target compound in a sample (see also copending United States
20 Patent Application No. 08/487,425, filed June 7, 1995, entitled "Enzyme Linked Oligonucleotide Assays (ELONAS)," United States Patent Application No. 08/479,729, filed June 7, 1995, entitled "Use of Nucleic Acid Ligands in Flow Cytometry," and United States Patent Application No. 08/628,356, filed April 5, 1996, entitled "Method for Detecting a Target Compound in a Substance Using a
25 Nucleic Acid Ligand"). United States Patent Application Serial No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Ligand Complexes," describes the use of oligonucleotides conjugated to lipophilic compounds or non-immunogenic, high molecular weight compounds to modulate the activity or pharmacokinetic behavior of the oligonucleotides. Conjugation has also been used to make oligonucleotide dimers
30 and to attach oligonucleotides to multimeric platforms. (Jones *et al.* (1995) J. Med. Chem. 38:2138).

Several chemical methods exist to accomplish such conjugations. (For a review, see Goodchild (1990) *Bioconjugate Chemistry* 1:165-187). The presence of a chemically reactive functional group, such as an amine or thiol, at the 5'-terminus of an oligonucleotide allows selective attachment of various conjugates, including reporter groups (Smith *et al.* (1987) *Methods Enzymol.* 155:260-301; Sproat *et al.* (1987) *Nucleic Acids Res.* 15:6181-6196; Mori *et al.* (1989) *Nucleosides & Nucleotides* 8:649; Sinha *et al.* (1988) *Nucleic Acids Res.* 16:2659) and peptide epitopes (Tung *et al.* (1991) *Bioconjugate Chem.* 2:464-465; Bruick *et al.* (1996) *Chem. Biol.* 3:49-56). Oligodeoxynucleotides containing a terminal amino functionality have been utilized for the construction of bioconjugates with novel properties. In some of the more common methods of synthesizing these bioconjugates, a primary, aliphatic amino group is incorporated at the 5'-terminus of the oligonucleotide in the final step of the assembly of a synthetic oligonucleotide (Tung *et al.* (1991) *Bioconjugate Chem.* 2:464-465; Smith *et al.* (1987) *Methods Enzymol.* 155:260-301). A commercial reagent (actually a series of such linkers having various lengths of polymethylene connectors) for linking to the 5' terminus of an oligonucleotide is 5'-Amino-Modifier C6. These reagents are available from Glen Research Corp (Sterling, VA). These compounds have been used by Krieg (Krieg *et al.* (1971) *Antisense Res. and Dev.* 1:161) to link fluorescein to the 5'-terminus of an oligonucleotide. Since many macromolecules of interest are hydrophilic, these reactions generally are done in water, requiring large excesses of reagent to overcome the competing hydrolysis. Usually the amine on the oligonucleotide is added to the terminus of the molecule and must compete with free amine and alcohol on the fully deprotected oligonucleotide if this modification is done post-synthetically.

In another common method of conjugating oligonucleotides to other molecular entities, particularly detector molecules, the molecular entity is converted into a phosphoramidite, which is then added to the free alcohol of the full length oligonucleotide which is attached to a solid support (Theison *et al.* (1992) *Tetrahedron Lett.* 33:5033-5036). This method is less than ideal due to the air and water sensitivity of the phosphoramidite, as well as, the fact that the molecule can only be added to the terminus of the oligonucleotide. Furthermore, many detector

molecules are not compatible with this method due to the harsh conditions normally needed to fully deprotect and release the oligonucleotide from the support. A third method of conjugating oligonucleotides to other molecules is the coupling of an alkylthio derivatized oligonucleotide with a α -haloacetyl or with a malcimide containing compound. (Jones *et al.* (1995) J. Med. Chem. 38:2138).

5 An alternative method for the synthesis of oligodeoxynucleotides terminated by 5'-amino-5'-deoxythymidine has been described (Bruick *et al.* (1997) Nucleic Acids Res. 25:1309-1310). This method uses a DNA template to direct the ligation of a peptide to an oligonucleotide, in which the peptide is presented by a second oligonucleotide in the form of a reactive thioester-linked intermediate.

10 The preparation of PEG-oligonucleotide conjugates is described by Goodchild (1990) Bioconjugate Chem. 1:165 and Zalipsky (1995) Bioconjugate Chem. 6:150). The preferred solvent for macromolecule conjugation reactions is an aqueous buffer. Most conjugation chemistry methods must be carried out at high pH and therefore, suffer severely from competing hydrolysis reactions. In addition, most conjugation reactions display poor chemoselectivity.

The preparation of conjugates of macromolecules is not limited to oligonucleotide conjugates. Proteins and peptides play a critical role in virtually all biological processes, functioning as enzymes, hormones, antibodies, growth factors, ion carriers, antibiotics, toxins, and neuropeptides. Proteins and peptides comprise a prominent class of pharmaceuticals. Conjugation of proteins and peptides to detector molecules or other macromolecules such as PEG is also a common practice. Conjugates of oligonucleotides with peptides having specific functions can be useful for various applications. Examples include the use of a nuclear transport signal peptide to direct intracellular trafficking (Eritja *et al.* (1991) Tetrahedron 47: 4113-4120); a hydrophobic peptide (Juby *et al.* (1991) Tetrahedron Lett. 32:879-822) or polylysine (Leonetti *et al.* (1991) Bioconjugate Chem. 1:149-153) to increase cell penetrability, and polylysine to provide multiple attachment sites for nonradioactive reporting probes (Haralambidis *et al.* (1987) Tetrahedron Lett. 28:5199-5202; Haralambidis *et al.* (1990) Nucleic Acids Res. 18:493-499).

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Cycloaddition reactions can be defined as any reaction between two (or more) moieties (either intra or intermolecular) where the orbitals of the reacting atoms form a cyclic array as the reaction progresses (typically in a concerted fashion although intermediates may be involved) along the reaction coordinate leading to a product.

- 5 The orbitals involved in this class of reactions are typically π systems although certain σ orbitals can also be involved. The number of electrons associated with this type of reaction are of two types; $4n+2$ and $4n$, where $n = 1, 2, 3, 4$ etc. Typical examples of cycloaddition reactions include Diels-Alder cycloaddition reactions, 1,3-dipolar cycloadditions and $[2+2]$ cycloadditions.

- 10 The Diels-Alder reaction, by far the most studied cycloaddition, is a cycloaddition reaction between a conjugated diene and an unsaturated molecule to form a cyclic compound with the π -electrons being used to form the new σ bonds. The Diels-Alder reaction is an example of a $[4 + 2]$ cycloaddition reaction, as it involves a system of 4- π electrons (the diene) and a system of 2- π electrons (the
- 15 dienophile). The reaction can be made to occur very rapidly, under mild conditions, and for a wide variety of reactants. The Diels-Alder reaction is broad in scope and is well known to those knowledgeable in the art. A review of the Diels-Alder reaction can be found in "Advanced Organic Chemistry" (March, J., ed.) 761-798 (1977) McGraw Hill, NY, which is incorporated herein by reference.

- 20 It has been discovered that the rate of Diels-Alder cycloaddition reactions is enhanced in aqueous solvents. (Rideout and Breslow (1980) J. Am. Chem. Soc. 102:7816). (A similar effect is also seen with 1,3-dipolar cycloaddition reactions (Engberts (1995) Tetrahedron Lett. 36:5389). This enhancement is presumably due to the hydrophobicity of the diene and dienophile reactants. (Breslow (1991) Acc.
- 25 Chem. Res. 24:159). This effect extends to intramolecular Diels-Alder reactions. (Blokzijl *et al.* (1991) J. Am. Chem. Soc. 113:4241). Not only is the reaction rate accelerated in water, but several examples of an increased endo/exo product ratio are also reported. (Breslow and Maitra (1984) Tetrahedron Lett. 25:1239; Lubineau *et al.* (1990) J. Chem. Soc. Perkin Trans. I, 3011; Grieco *et al.* (1983) Tetrahedron Lett.
- 30 24:1897). Salts which increase the hydrophobic effect in water, such as lithium chloride (Breslow *et al.* (1983) Tetrahedron Lett. 24:1901) and also monovalent

phosphates (Pai and Smith (1995) J. Org. Chem. 60:3731) have been observed to further accelerate the rate of 4+2 cycloadditions.

The synthetic potential of the Diels-Alder reaction in aqueous solvents is gaining increasing attention. It has been demonstrated that simple dienes, such as sodium 3,5-hexadienoate and sodium 4,6-heptadienoate readily undergo Diels-Alder reactions in water with a variety of dienophiles at ambient temperature. (Grieco *et al.* (1983) J. Org. Chem. 48:3137). The otherwise difficult cycloaddition of dimethyl acetylenedicarboxylate to an electron deficient furan proceeds under very mild conditions in water with very good yields. (Saksena *et al.* (1993) Heterocycles 35:129). The scope of the reaction has been extended to cycloaddition of iminium salts, generated in situ from an ammonium salt and formaldehyde to dienes. (Grieco and Larsen (1985) J. Am. Chem. Soc. 107:1768). This work inspired the exploration of the corresponding reaction of iminium salts of amino acids with dienes which proceeds with high stereoselectivity. (Grieco *et al.* (1986) Tetrahedron Lett. 27:1975; Grieco and Bahsas (1987) J. Org. Chem. 52:5745; Waldmann (1989) Liebigs Ann. Chem., 231-238; Waldmann and Braun (1991) Liebigs Ann. Chem., 1045-1048). The scope of this reaction has also been extended to more complex aldehydes by use of lanthanide(III) trifluoromethanesulfonates as catalysts. (Yu *et al.* (1996) Tetrahedron Lett. 37:2169).

In copending PCT Application Serial No. PCT/US96/16668, filed on October 17, 1996, designating the United States, entitled "Method for Solution Phase Synthesis of Oligonucleotides" and United States Application Serial No. 08/843,820 entitled "Method for Solution Phase Synthesis of Oligonucleotides," both of which are incorporated herein by reference in their entirety, the Diels-Alder cycloaddition reaction is shown to be an ideal method for anchoring oligonucleotides onto resins. Resins derivatized with a diene or dienophile are reacted with an oligonucleotide derivatized with a dienophile or diene, respectively, to yield the Diels-Alder cycloaddition product. In particular, Diels-Alder reactions between oligonucleotides derivatized with a diene and polymeric resins derivatized with maleimide groups and with phenyl-triazoline-dioncs (PTAD) are described. The resulting resins can be used as affinity chromatography resins.

The present invention illustrates that cycloaddition reactions, such as the Diels-Alder reaction and 1,3-dipolar cycloaddition reactions, are an ideal replacement for current methods of conjugating macromolecules with other molecular moieties. The Diels-Alder reaction, in particular, is an ideal method for covalently linking large water soluble macromolecules with other compounds as the reaction rate is accelerated in water and can be run at neutral pH. (Rideout and Breslow (1980) J. Am. Chem. Soc. 102:7816). Additionally, the nature of the reaction allows post-synthetic modification of the hydrophilic macromolecule without excess reagent or hydrolysis of the reagent. With respect to conjugation to oligonucleotides, this technology has been aided by the ability to efficiently synthesize 2'-O-diene-nucleosides, which allows the conjugation site to be varied throughout the oligonucleotide or the option of having multiple conjugation sites.

BRIEF SUMMARY OF THE INVENTION

The present invention describes a novel, chemoselective and highly efficient method for derivatizing or conjugating macromolecules with other molecular entities. Specifically, the present invention describes the use of cycloaddition reactions, including but not limited to Diels-Alder reactions, 1,3-dipolar cycloaddition reactions and [2+2] cycloaddition reactions, for the chemoselective and efficient derivatization or conjugation of macromolecules with other molecular entities. Thus, a macromolecule bearing a moiety capable of undergoing a cycloaddition reaction, is reacted with another molecular entity bearing a moiety capable of undergoing a cycloaddition reaction with the moiety attached to the macromolecule to yield via a cycloaddition reaction efficient conjugation of the molecular entity to the macromolecule.

In a preferred embodiment the cycloaddition reaction is a Diels-Alder reaction. Thus, a macromolecule bearing either a diene or dienophile moiety is reacted with another molecular entity bearing either a dienophile or a diene moiety, respectively, to yield via a cycloaddition reaction efficient conjugation of the molecular entity to the macromolecule.

In one embodiment the macromolecule is an oligonucleotide. Thus, an oligonucleotide bearing either a diene modified nucleoside or non-nucleoside phosphate diester group, or a dienophile modified nucleoside or non-nucleoside phosphate diester group is reacted with a molecular entity bearing either a dienophile or a diene moiety. Diels-Alder cycloaddition leads to efficient conjugation of the oligonucleotide with the molecular entity. The molecular entity can be any molecule, including another macromolecule which can be derivatized with a dienophile, diene or other moiety capable of undergoing a cycloaddition reaction. Examples of molecular entities include but are not limited to other macromolecules, polymers or resins, such as polyethylene glycol (PEG) or polystyrene, diagnostic detector molecules, such as fluorescein, coumarin or a metal chelator.

The Diels-Alder cycloaddition between a diene modified oligonucleotide and a dienophile modified oligonucleotide (or any cycloaddition reaction between suitably derivatized oligonucleotides and their reacting partners) results in efficient and specific formation of oligonucleotide homo-dimers and hetero-dimers. In addition, dimers or multimers of oligonucleotides can be prepared efficiently by reaction of two or more diene-modified oligonucleotides with a linker group bearing two or more dienophile moieties. Conventional activated acid linking chemistries do not allow for efficient dimerization or multimerization, since they are limited by competing hydrolysis of the activated acid reagents by water.

This invention includes a reaction scheme for producing a wide variety of conjugated macromolecules using cycloaddition reactions as typified by the Diels-Alder cycloaddition reaction and 1,3-dipolar cycloaddition reactions. The method of this invention can be extended to the conjugation of any macromolecule with another molecular entity, including but not limited to nucleic acids, proteins, peptides carbohydrates, polysaccharides, glycoproteins, lipids, hormones, drugs or prodrugs.

The method of this invention can be extended to all $4n$ and $4n+2$ cycloadditions (where $n = 1, 2, 3, 4$, etc.). This includes, but is not limited to Diels-Alder cycloadditions, 1,3-dipolar cycloadditions, ene cycloaddition reactions, and $[2+2]$ ($4n$ type) cycloadditions such as ketene additions and photochemical $2+2$ additions.

Also included in this invention are any novel conjugated macromolecules produced by the method of this invention.

BRIEF DESCRIPTION OF THE FIGURES

5 FIGURE 1 illustrates graphically the percent completion of the Diels-Alder reactions of compounds 3 (○), 4 (◻) and 6 (◇) with N-ethylmaleimide as a function of time. (Example 1).

10 FIGURE 2 illustrates graphically the percent completion of the Diels-Alder reactions of compounds 5 (○) and 6 (◻) with N-ethylmaleimide as a function of time. (Example 1).

15 FIGURE 3 illustrates graphically the dimerization rate for the Diels-Alder reaction of 5'-DMT-thymidine 3'-hexadiene-(2-cyanoethyl)phosphite with 4,4'-dimaleimidodiphenylmethane. The amount of product was calculated as a percent of unreacted starting material, mono Diels-Alder product (◻) and dimer (■).

20 FIGURE 4 illustrates the reverse phase HPLC of the crude 5'-hexadienoxy-phosphate-DNA (12). The peak at 23 minutes (42% by area integration) corresponds to the full-length oligonucleotide product 12.

25 FIGURE 5 illustrates the reverse phase HPLC of the crude polyethylene glycol-DNA conjugate (14). The peak at 9.5 minutes (80% by area integration) corresponds to the PEG-oligo conjugate product 14.

DETAILED DESCRIPTION OF THE INVENTION

30 The present invention includes a novel method for conjugating macromolecules with other molecular entities. Specifically, the present invention describes the use of cycloaddition reactions, in particular the Diels-Alder cycloaddition reaction for the chemoselective and efficient derivatization or conjugation of macromolecules with molecular entities. Thus, a macromolecule

bearing either a diene or dienophile moiety is reacted with another molecular entity bearing either a dienophile or a diene moiety, respectively, to yield via Diels-Alder reaction efficient conjugation of the molecular entity to the macromolecule.

- The macromolecule can be any large organic molecule which bears or can be derivatized to bear a moiety capable of undergoing a cycloaddition reaction, including but not limited to nucleic acids, oligonucleotides, proteins, peptides, carbohydrates, polysaccharides, glycoproteins, lipids, hormones, drugs or prodrugs. The molecular entity can be any molecule, including another macromolecule, which bears or can be derivatized to bear a moiety capable of undergoing a cycloaddition reaction.
- 10 Examples of molecular entities include but are not limited to other macromolecules, including antibodies, polymers or resins, such as polyethylene glycol (PEG) or polystyrene, diagnostic detector molecules, such as fluorescein, biotin, coumarin or a metal chelator. In a preferred embodiment the cycloaddition reaction is a Diels-Alder reaction and the macromolecule and molecular entity are derivatized with a diene or
- 15 dienophile, respectively.

Certain terms used to describe the invention herein are defined as follows:

- "Nucleoside" as used herein is defined as a modified or naturally occurring deoxyribonucleoside or ribonucleoside or any chemical modifications thereof. Modifications of the nucleosides include, but are not limited to, 2'-, 3'- and 5'-position sugar modifications, 5- and 6-position pyrimidine modifications, 2-, 6- and 8-position purine modifications, modifications at exocyclic amines, substitution of 5-bromouracil, and the like. Nucleosides can be suitably protected and derivatized to enable oligonucleotide synthesis by methods known in the field, such as solid phase automated synthesis using nucleoside phosphoramidite monomers, H-phosphonate
- 25 coupling or phosphate triester coupling.

- "Nucleotide" as used herein is defined as a modified or naturally occurring deoxyribonucleotide or ribonucleotide. Nucleotide is a nucleoside as defined above having one or several phosphates or substituted phosphates attached at the 5'-, 2'- or 3'-positions. Nucleotides typically include purines and pyrimidines, which include
- 30 thymidine, cytidine, guanosine, adenine and uridine.

"**Oligonucleotide**" refers to a polynucleotide formed from a plurality of linked nucleotide units as defined above. The nucleotide units each include a nucleoside unit linked together via a phosphate linking group. The term oligonucleotide also refers to a plurality of nucleotides that are linked together via linkages other than phosphate linkages such as phosphorothioate linkages. The oligonucleotide may be naturally occurring or non-naturally occurring. In a preferred embodiment the oligonucleotides of this invention have between 1-1,000 nucleotides.

For the purposes of this invention "**nucleobase**" will have the following definition. A nucleobase is a purine or a pyrimidine base. Nucleobase includes all purines and pyrimidines currently known to those skilled in the art or any chemical modifications thereof. The purines are attached to the ribose ring through the nitrogen in the 9 position of the purine ring and the pyrimidines are attached to the ribose ring through the nitrogen in the 1 position of the pyrimidine ring. The pyrimidine can be modified at the 5- or 6- position of the pyrimidine ring and the purine can be modified at positions 2-, 6- or 8- of the purine ring. Certain modifications are described in copending United States Patent Applications Serial Nos. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2'- Modified Nucleosides by Intramolecular Nucleophilic Displacement" and 08/458,421, filed June 2, 1994, entitled "Palladium Catalyzed Nucleoside Modifications Methods Using Nucleophiles and Carbon Monoxide" and United States Patent No. 5,428,149, entitled "Method for Palladium Catalyzed Carbon-Carbon Coupling and Products" which are herein incorporated by reference in their entirety. More specifically a nucleobase includes, but is not limited to, uracil, cytosine, N4-protected cytosine, 4-thiouracil, isocytosine, 5-methyluracil (thymine), 5-substituted uracils, adenine, N6-protected adenine, guanine, N2-protected guanine, 2,6-diaminopurine, halogenated purines as well as heterocycles meant to mimic the purine or pyrimidine ring, such as imidazole.

"**Nucleic acid ligand**" as used herein is a nucleic acid having a desirable action on a target. A desirable action includes, but is not limited to, binding of the target, catalytically changing the target, reacting with the target in a way which modifies/alters the target or the functional activity of the target, covalently attaching to the target as in a suicide inhibitor, facilitating a reaction between the target and/or

another molecule. In the preferred embodiment, the action is specific binding affinity for a target molecule, such target molecule being a three dimensional chemical structure other than a polynucleotide that binds to the nucleic acid ligand through a mechanism which predominantly depends on Watson/Crick base pairing or triple helix binding, wherein the nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule. In one embodiment, the nucleic acid ligand is a non-naturally occurring nucleic acid. In preferred embodiments of the invention, the nucleic acid ligands are identified by the SELEX methodology. Nucleic acid ligands includes nucleic acids that are identified from a candidate mixture of nucleic acids, said nucleic acid ligand being a ligand of a given target, by the method comprising a) contacting the candidate mixture with the target, wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture; b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and c) amplifying the increased affinity nucleic acids to yield a ligand-enriched mixture of nucleic acids.

"Nucleic acid" means either DNA, RNA, single-stranded or double-stranded and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil, backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

"Non-immunogenic, high molecular weight compound" is a compound of approximately 1000 Da or more that typically does not generate an immunogenic response. An immunogenic response is one that induces the organism to produce antibody proteins. Examples of non-immunogenic, high molecular weight compounds

include polyethylene glycol (PEG); polysaccharides, such as dextran; polypeptides, such as albumin; and magnetic structures, such as magnetite.

The term "**protein**" as used herein refers to a polymer of amino acids, chemically bound by amide linkages (CONH). An "**amino acid**" is defined as an
5 organic molecule containing both an amino group (NH₂) and a carboxylic acid (COOH). Specifically an "**amino acid**" is any compound of the general formula R⁵CH(NH₂)COOH (α -amino acid), wherein R⁵ is selected from the group consisting of H or any suitably protected known amino acid side chain or any chemical
10 modifications thereof. Suitable protection for amino acid side chains is known to those skilled in the art. As used herein the term "protein" includes peptides, polypeptides and proteins. In a preferred embodiment the proteins of this invention have between 1-500 amino acids.

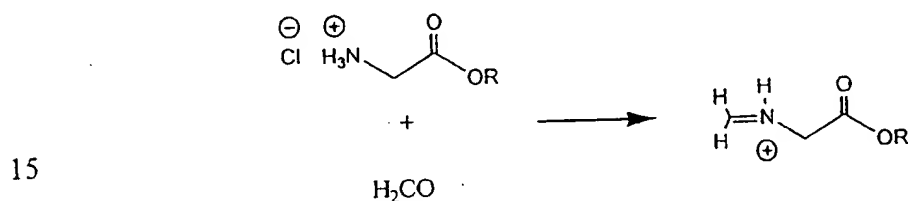
The term "**lipophilic compounds**" as used herein refers to compounds which have the propensity to associate with or partition into lipid and/or other materials or
15 phases with low dielectric constants, including structures that are comprised substantially of lipophilic components. Cholesterol, phospholipids, diacyl glycerols and dialkyl glycerols are examples of lipophilic compounds.

A "**diene**" is defined as a molecule bearing two conjugated double bonds. The diene may even be non-conjugated, if the geometry of the molecule is constrained so
20 as to facilitate a cycloaddition reaction (Cookson (1964) J. Chem. Soc. 5416). The atoms forming these double bonds can be carbon or a heteroatom or any combination thereof.

A "**dienophile**" is defined as a molecule bearing an alkene group, or a double bond between a carbon and a heteroatom, or a double bond between two heteroatoms.
25 The dienophile can be any group, including but not limited to, a substituted or unsubstituted alkene, or a substituted or unsubstituted alkyne. Typically, the dienophile is a substituted alkene of the formula C=C-Z, or Z'-C=C-Z, wherein Z and Z' are electron withdrawing groups independently selected from CHO, COR, COOH, COCl, COaryl, CN, NO₂, aryl, CH₂OH, CH₂Cl, CH₂NH₂, CH₂CN, CH₂COOH, halogen, or C=C. In certain cases the groups attached to the alkene unit can be
30 electron donating groups, including but not limited to phenyl rings, conjugated

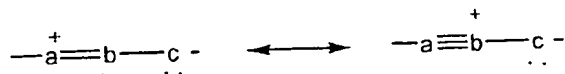
double bonds, alkyl groups, OMe groups or other X-alkyl moieties wherein X is an electron donating group (these type of dienophiles under go cycloadditions that are known generally as reverse electron demand cycloadditions). Other examples of dienophiles include compounds having the formula, $R_2C=X$, wherein X is a heteroatom, selected from the group consisting of oxygen, nitrogen, phosphorus and sulfur. For example, molecules bearing a primary amino group, such as amino acids or a lysine containing peptide, can be converted to efficient dienophiles by reaction with formaldehyde to yield their corresponding iminium salts as illustrated in Scheme 1. The latter undergo Diels-Alder cycloaddition with macromolecules bearing a diene group under mild conditions in aqueous solvents.

SCHEME 1

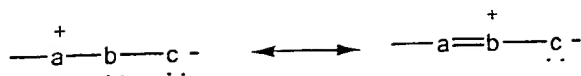


A "1,3-dipole" is defined as a compound that contains a consecutive series of three atoms, a-b-c, where atom a contains a sextet of electrons in its outer shell and atom c contains an octet with at least one unshared pair of electrons in its outer shell. Because molecules which have six electrons in the outer shell of an atom are typically unstable, the a-b-c atom example is actually one canonical structure of a resonance hybrid, where at least one other structure can be drawn. 1,3-dipoles can be divided into two main groups:

1) Systems in which one of the canonical forms has a double bond on the sextet atom (atom a) and the other canonical form has a triple bond on that atom:



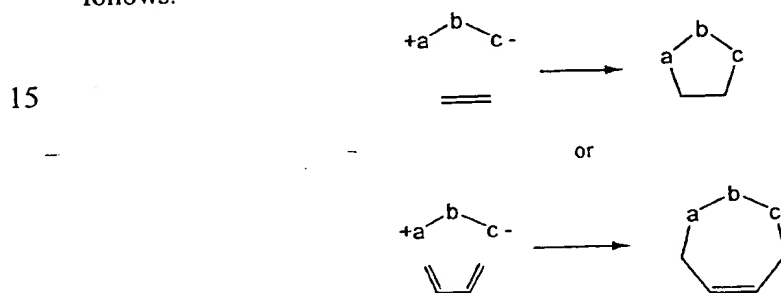
2) Systems where the dipolar canonical form has a single bond on the sextet atom (atom a) and the other canonical form has a double bond on that atom:



For a review of this reaction type see "Advanced Organic Chemistry". (March, J., ed.) 758-761 (1977) McGraw Hill, NY, and "Frontier Orbitals and Organic Chemical
 5 Reactions" (I. Fleming) 148-161 (1976) John Wiley and Sons, Ltd. Typical examples include, but are not limited to nitrile ylids, nitrile imines, nitrile oxides, diazoalkanes, azides, azomethine ylids, azomethine imines, nitrones, carbonyl ylids, carbonyl imines and carbonyl oxides.

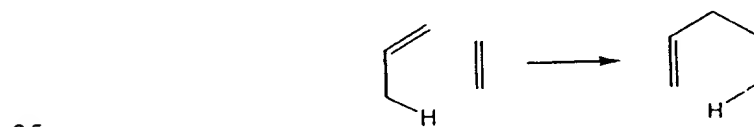
A "1,3-dipolarophile" is defined in the same manner as a "dienophile" or
 10 "diene" (as described above). The macromolecule can be attached to either (or both) the 1,3-dipole or the 1,3-dipolarophile.

A "1,3-dipolar cycloaddition reaction" can be generally represented as follows:



20

An "Ene reaction" can be generally represented as follows:



The reaction partners in an Ene reaction are referred to an "ene" and an "enophile."

An "enophile" is defined in the same manner as a "dienophile" (see the above description for dienophile). An "ene" can be any unsaturated group, including but not
 30 limited to, a substituted or unsubstituted alkene, or a substituted or unsubstituted alkyne. Typically, the "ene" is a substituted alkene of the formula X-C=CH-CH₂- or

$X'-C=CX-CH_X-$, wherein X and X' are electron donating groups. The macromolecule can be attached to either (or both) the ene component or the enophile component.

As used herein a "macromolecule" refers to a large organic molecule.

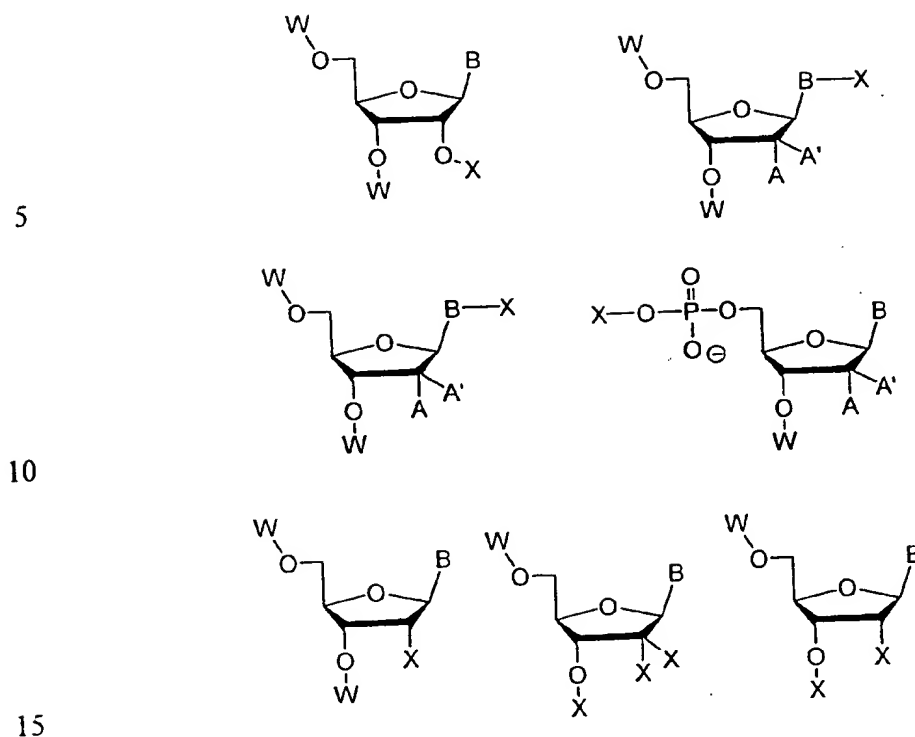
Examples of macromolecules include, but are not limited to nucleic acids, oligonucleotides, proteins, peptides, carbohydrates, polysaccharides, glycoproteins, lipids, hormones, drugs, or prodrugs.

"Bioconjugate" as defined herein refers to any macromolecule which has been derivatized with another molecular entity. "Bioconjugation" or "Conjugation" refers to the derivatization of a macromolecule with another molecular entity.

The "molecular entity" can be any molecule and can include a small molecule or another macromolecule. Examples of molecular entities include but are not limited to other macromolecules, polymers or resins, such as polyethylene glycol (PEG) or polystyrene, non-immunogenic high molecular weight compounds, fluorescent, chemiluminescent radioisotope and bioluminescent marker compounds, antibodies, biotin, diagnostic detector molecules, such as a maleimide derivatized fluorescein, coumarin, a metal chelator or any other modifying group. The terms bioconjugation and conjugation are used interchangeably throughout the Specification.

A "derivatized macromolecule" refers to a macromolecule that has been functionalized with a moiety capable of undergoing a cycloaddition reaction. A macromolecule that bears a moiety capable of undergoing a cycloaddition reaction without functionalization also falls within this definition. Examples of moieties capable of undergoing a cycloaddition reaction are defined below as X. In a preferred embodiment the macromolecule is functionalized with a diene or a dienophile. In a most preferred embodiment the dienophile is a maleimide and the diene is a hexadiene.

The "derivatized oligonucleotides" of this invention are generally represented by the following formulas:



wherein

B is a nucleobase;

A and A' are 2'-sugar substituents;

W is independently selected from the group consisting of an oligonucleotide having
20 between 1-1000 nucleobases. X or H; and

X is a diene, dienophile, 1,3-dipole, 1,3-dipolarophile, ene, enophile or other moiety
capable of undergoing a cycloaddition reaction additionally, when X is attached to the
nucleobase B it can be attached to a carbon atom, an exocyclic nitrogen or an
exocyclic oxygen.

25 In a preferred embodiment of the invention:

A and A' are independently selected from the group consisting of H, ^2H , ^3H , Cl, F,
OH, NHOR^1 , NHOR^3 , NHNHR^3 , NHR^3 , $=\text{NH}$, CHCN , CHCl_2 , SH, SR^3 , CFH_2 , CF_2H ,
 CR_2^2Br , $-(\text{OCH}_2\text{CH}_2)_n\text{OCH}_3$, OR^4 and imidazole (see United States Patent Application
Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of
30 2' Modified Pyrimidines Intramolecular Nucleophilic Displacement." which is
incorporated herein by reference);

- R^1 is selected from the group consisting of H and an alcohol protecting group;
 R^2 is selected from the group consisting of =O, =S, H, OH, CCl_3 , CF_3 , halide, optionally substituted C_1 - C_{20} alkyl (including cyclic, straight chain, and branched), alkenyl, aryl, C_1 - C_{20} acyl, benzoyl, OR^4 and esters;
- 5 R^3 is selected from the group consisting of R^2 , R^4 , CN, $C(O)NH_2$, $C(S)NH_2$, $C(O)CF_3$, SO_2R^4 , amino acid, peptide and mixtures thereof;
 R^4 is selected from the group consisting of an optionally substituted hydrocarbon (C_1 - C_{20} alkyl, C_2 - C_{20} alkenyl, C_2 - C_{20} alkynyl), an optionally substituted heterocycle, t-butyltrimethylsilyl ether, triisopropylsilyl ether, nucleoside, carbohydrate, fluorescent
- 10 label and phosphate; most preferably A is selected from the group consisting of H, OH, NH_2 , Cl, F, $NHOR^3$, OR^4 , $OSiR^4_3$. (See United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of 2' Modified Pyrimidines Intramolecular Nucleophilic Displacement," filed June 22, 1994); and
- 15 X includes but is not limited to an alkyl or substituted alkyl group bearing a conjugated diene unit, an alkoxy or substituted alkoxy group bearing a conjugated diene unit, $CH_2=CHCH=CHCH_2CH_2O-$, maleimide substituted alkoxy groups, dienophile substituted alkoxy groups, alkoxy groups, an alkylamino or substituted alkylamino group bearing a conjugated diene unit, maleimide substituted alkylamino
- 20 groups or substituted alkylamino groups, an alkylamino group or substituted alkylamino group bearing a dienophile moiety, a nitrile ylid, nitrile imine, nitrile oxide, diazoalkane, azide, azomethine ylid, azomethine imine, nitron, carbonyl ylid, carbonyl imine and carbonyl oxide. The alkyl groups on the above listed substituents can have between 1-50 carbons, preferably 1-30 carbons.
- 25 As used herein a "crosslinking molecule" is a molecular entity that connects two or more molecular entities through covalent interactions. More specifically a "crosslinking molecule" is a multifunctional molecule that can be used to derivatize a macromolecule with a diene, dienophile or other moiety capable of undergoing a cycloaddition reaction or a molecule to be conjugated to a macromolecule with a
- 30 diene, dienophile, or other moiety capable of undergoing a cycloaddition reaction.

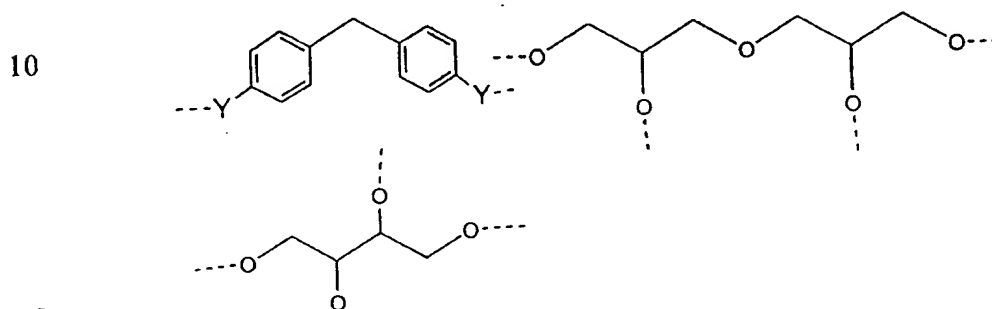
The crosslinking molecules of this invention are generally represented by the following formulas:



5 wherein X is a diene or dienophile, as defined above;

n is an integer from 1-20; and

L is a linker which includes, but is not limited to, compounds of the following general formula:



wherein

Y is selected from NH, O, NH(CO)O, NH(CS)O, NH(CO)NH, NH(CO), S-S-S- or Si(R)₂, wherein

R is selected from alkyl, aryl, substituted alkyl or substituted aryl.

20 Other obvious substitutions for the substituents described above are also included within the scope of this invention, which is not limited to the specific, but rather the generalized formula of reaction.

"Therapeutic Agent" means a compound which is used in the treatment of diseases and disorders.

25 "Diagnostic Agent" means a bioconjugate which can be used for detecting the presence or absence of and/or measuring the amount of target in a sample. Detection of the target molecule is mediated by its binding to a nucleic acid component of a bioconjugate specific for that target molecule. The bioconjugate can be labeled, for example radiolabeled, to allow qualitative or quantitative detection.

30 "Improved pharmacokinetic properties" means that a bioconjugate shows a longer circulation half-life *in vivo* relative to a nucleic acid that is not part of a

bioconjugate, or has other pharmacokinetic benefits such as improved target to non-target concentration ratio.

Cycloaddition reactions, particularly Diels-Alder reactions, are uniquely suited as a general method for the conjugation of macromolecules to each other, to
5 diagnostic detectors or to other modifying groups. The cycloaddition of a diene to a dienophile is highly chemoselective and only a suitably electronically configured diene and dienophile pair will react. The reaction proceeds under mild conditions in a reasonable time-frame. Macromolecules such as nucleic acids, oligonucleotides, proteins, peptides, carbohydrates, polysaccharides, glycoproteins and lipids generally
10 do not contain moieties that can undergo such a cycloaddition reaction. Thus, by specific introduction of a diene and dienophile reaction partner, macromolecule conjugation, derivatization, or multimerization becomes possible with unprecedented specificity.

The high selectivity of a diene or dienophile for reaction with the
15 corresponding dienophile or diene, respectively eliminates the need to protect functional groups during the synthesis of macromolecules such as oligonucleotides or peptides. This is a tremendous practical advantage over other functional groups used for conjugation in macromolecule synthesis, in which the limited selectivity of the protection chemistry often determines the conjugation yields. Additionally, the diene
20 and dienophiles are not susceptible to the side-reactions typically encountered in conjugation methods. Because, they do not undergo hydrolysis or solvolysis reactions, these reactions can be performed in aqueous media at near stoichiometric concentrations and thus conserve precious reagent. The lack of such side reactions allows dimerization and multimerization of macromolecules in unprecedented yields
25 and purities. The Diels-Alder cycloaddition reaction is accelerated by aqueous solvents and therefore uniquely suited for the derivatization or conjugation of hydrophilic macromolecules. Finally, this conjugation method is much less pH sensitive than most known alternatives.

In one embodiment of the present invention the macromolecule is an
30 oligonucleotide. The solvent of choice for the derivatization of oligonucleotides is water, due to the highly anionic nature of these molecules. Thus, an optimal reaction

for the conjugation of such groups to oligonucleotides proceeds readily in water, and displays no side reactions with water, such as hydrolysis of any of the reactants. Based on these criteria for optimal and specific introduction of substituents to oligonucleotides, this disclosure describes the use of Diels-Alder cycloadditions for the chemoselective and efficient modification of oligonucleotides. Thus an oligonucleotide bearing either a diene modified nucleoside or non-nucleoside phosphate diester group, or a dienophile modified nucleoside or non-nucleoside phosphate diester group can be reacted with a molecular entity bearing either a dienophile or a diene moiety, respectively.

10 The diene or dienophile moiety can be incorporated into the oligonucleotide at any position in the chain, for instance by introduction of a 5-(3,5-hexadiene) 2'-deoxyuridine nucleoside (see United States Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2'-modified Nucleosides by Intramolecular Nucleophilic Displacement," which is
15 incorporated herein by reference in its entirety). Alternatively, the diene or dienophile moiety can be introduced as the 2'-O-(3,5-hexadiene)uridine nucleoside. A diene moiety can also be introduced to the oligonucleotide as a diene-bearing non-nucleoside phosphoramidite, such as 3,5-hexadiene-N,N-diisopropyl-2-cyanoethyl phosphoramidite. Reaction of the diene modified oligonucleotide, such as a 5'-
20 terminal 3,5-hexadienephosphate oligonucleotide, with the dienophile modified reactant, such as malcimidopolyethylene-glycol methylether, leads to efficient conjugation of the oligonucleotide.

 The method of this invention can be extended to the bioconjugation of any macromolecule that can be derivatized with a diene, dienophile or other reactive group
25 capable of undergoing a cycloaddition reaction without limitation. For example, the method can be extended to the conjugation of peptides and proteins with other molecular entities. A peptide or protein that contains an amino acid building block which has been derivatized with a diene or dienophile, such as O-3,5-hexadiene-tyrosine or serine, or N-maleimidolysine, can be conjugated to another molecular
30 entity including, but not limited to, another peptide, an oligonucleotide, nucleic acid, carbohydrate, detector molecule etc. without limitation. Natural macromolecules such

as proteins can be derivatized with a diene or dienophile bearing heterobifunctional crosslinking reagent, such as the NHS ester of 3-(4-maleimidophenyl)-propionic acid (Pierce), which allows subsequent conjugation to a macromolecule or diagnostic detector molecule bearing a corresponding diene or dienophile group.

5 The high chemoselectivity of cycloaddition reactions, particularly the Diels-Alder reaction allows their exploitation for dimerization of macromolecules. When two active macromolecules are combined to a single molecular entity, their activity can be enhanced exponentially. Homobifunctional dimers, comprised of two identical macromolecules, or heterobifunctional dimers, comprised of two molecules with
10 different activity, can be assembled with high specificity and high yield by cycloaddition reaction, particularly by Diels-Alder cycloaddition. For example, an oligonucleotide bearing a diene moiety, such as a 3,5-hexadiene or a cyclopentadiene group, either at the 5'- or 3'-terminus or at a 2'-position or C-5 position anywhere in the sequence can be covalently linked to a second oligonucleotide bearing a
15 dienophile moiety, such as a maleimide or acrylamide group. Such oligonucleotides bearing a diene or dienophile group can react directly with an oligonucleotide bearing a corresponding dienophile or diene group to form dimers. Depending on the point of attachment of the diene or dienophile group, dimers of oligonucleotides can be obtained in either a 5'-3', 5'-5', 3'-3', 5'-internal, 3'-internal, or internal-internal
20 orientation. Alternatively, such oligonucleotides can react with a crosslinking molecule containing either two or more diene or dienophile groups to form dimers or multimers. Dimerization of oligonucleotides using the method of this invention is illustrated in Examples 2, 4 and 6 below.

Polyethylene glycol is often conjugated to macromolecules to reduce their
25 immunogenicity and to increase their residence time *in vivo*. The bioconjugation method of this invention allows derivatization of macromolecules, such as oligonucleotides or peptides, bearing a diene, dienophile or other reactive group capable of undergoing a cycloaddition reaction with another polymer or resin, such as polyethylene glycol or polystyrene bearing one or several corresponding diene,
30 dienophile or other groups capable of undergoing cycloaddition reactions.

Macromolecules or molecular entities bearing a dienophile group or other reactive group capable of undergoing a cycloaddition reaction can also undergo cycloadditions to unsaturated lipids bearing diene units or other reactive units capable of undergoing a cycloaddition reaction. The resulting lipid conjugates are useful for anchoring the macromolecules or detector molecules in lipid phases such as micelles or liposomes.

As stated above, conjugation by cycloaddition reactions, particularly the Diels-Alder cycloaddition is not limited to the reaction of macromolecules with each other. It is also extremely useful for the selective derivatization of a macromolecule, such as, an oligonucleotide or peptide, bearing one or several dienes, dienophiles or other reactive groups capable of undergoing a cycloaddition reaction, with a diagnostic detector molecule bearing a diene, dienophile or other group capable of undergoing cycloaddition, such as a maleimide derivatized fluorescein or a maleimide derivatized metal chelator.

The bioconjugation method described herein is also useful to expand the properties and functionality of transcribed oligonucleotides, specifically for the SELEX drug discovery process. The SELEX method is described in United States Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by EXponential Enrichment," now abandoned; United States Patent Application Serial No. 07/714,131, filed June 10, 1991, entitled "Nucleic Acid Ligands," now United States Patent No. 5,475,096; United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled "Methods of Identifying Nucleic Acid Ligands," now United States Patent No. 5,270,163 (See also PCT Application Publication No. WO 91/19813). These applications, each specifically incorporated herein by reference, are collectively called the SELEX Patent Applications.

The modification of oligonucleotides by selective conjugation of substituents to multiple modified nucleoside constituents is not limited to oligonucleotides generated by transcription. Nucleoside analogs bearing a reaction center, such as a diene or dienophile, capable of selective reaction with a complementary reactant, such as a dienophile or diene in a cycloaddition reaction, can also be synthetically

incorporated into oligonucleotides. A 2'-hexadienecoxyuridine monomer, for example, suitably derivatized to the 5'-protected 3'-phosphoramidite by standard methods, can be incorporated into an oligonucleotide by standard automated solid phase synthesis. This generates a synthetic oligonucleotide bearing multiple internal
5 diene substituents. This oligonucleotide can be conjugated to multiple substituents bearing dienophile groups, such as maleimido polyethylene glycol. Thus, a polyethylene glycol coated synthetic oligonucleotide is generated.

Example 1 illustrates the feasibility of using the Diels-Alder reaction for the bioconjugation of macromolecules to other molecular entities. In this example the
10 Diels-Alder reactions of several hexadiene phosphate nucleosides with N-ethylmaleimide is described. The reactions proceed rapidly, in high yields with approximately 1.2 equivalents of maleimide in pure water or 20% iPrOH in water.

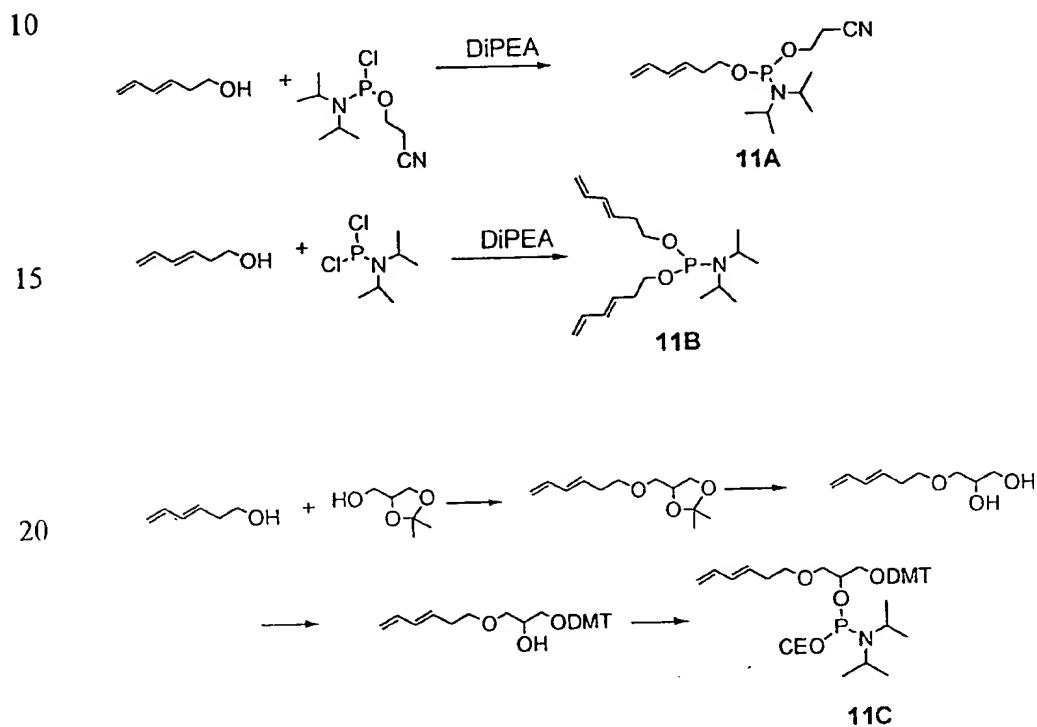
Example 2 (Scheme 6) describes the dimerization of 5'-DMT-thymidine 3'-hexadiene-(2-cyanoethyl)phosphite (7) with 4,4'-dimaleimidodiphenylmethane
15 dimaleimide (8). Figure 3 illustrates graphically the amount of mono-Diels-Alder cycloaddition product (mono conjugate) and dimer conjugate present (9) in the reaction mixture over a 22 hour period of time. The amount of the two products was calculated as a percent of unreacted starting material, mono conjugate and dimer conjugate present in the reaction mixture. This graph shows, as expected, that the
20 mono conjugate attains a relatively stable concentration and slowly declines as the dimer conjugate is formed.

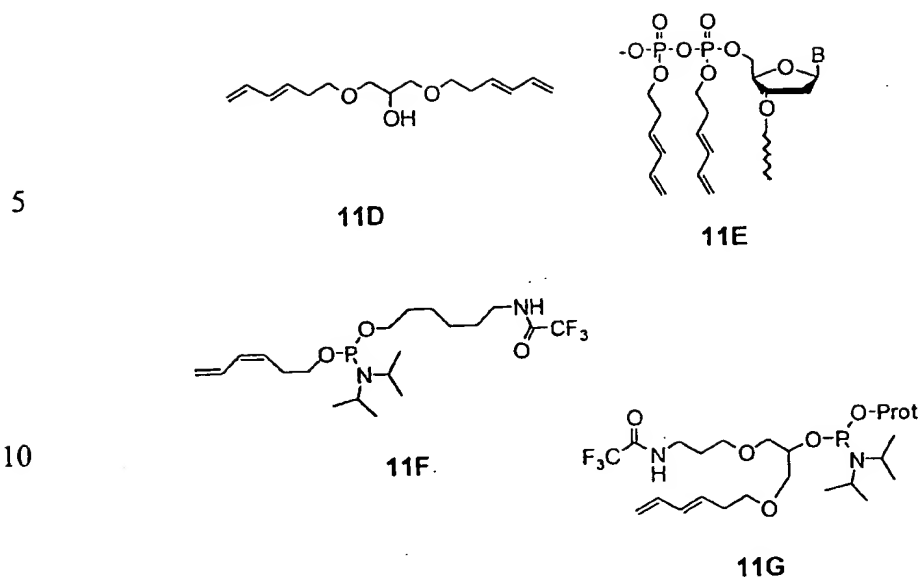
Bioconjugation of oligonucleotides depends on the ability to modify the oligonucleotide with a moiety capable of undergoing cycloaddition reaction. One approach is to incorporate the reactive moiety, such as the diene or dienophile into
25 either the sugar or base of a nucleoside, as illustrated in Example 2. A second approach is to prepare a phosphoramidite containing the reactive moiety, which can then undergo coupling and oxidation. The reactive moiety must be able to survive or become unmasked by any deprotection steps. The phosphoramidite may be a 5'-O-terminus for an oligonucleotide chain, or it may have another protected alcohol that
30 may be deprotected for further chain elongation. Examples of compounds which can be used to prepare phosphoramidites containing a diene moiety are set forth in

Scheme 2. The synthesis of compounds **11A** and **11B** is set forth in Example 3 below.

The 3'-O or 5'-O-terminus may also be modified with a charged bis(diene) phosphate via the synthesis of a bis(diene)-ol such as **11D** followed by conversion to a phosphoramidite via normal phosphoramidite syntheses. This may also be accomplished with the synthesis of a diphosphate ester **11E**. It is also possible that a "heterobifunctional" phosphoramidite, exemplified by **11F** or **11G**, may be a useful compound for crosslinking or a selective two step bioconjugation to different conjugates. Prot represents any standard alcohol protecting group.

SCHEME 2





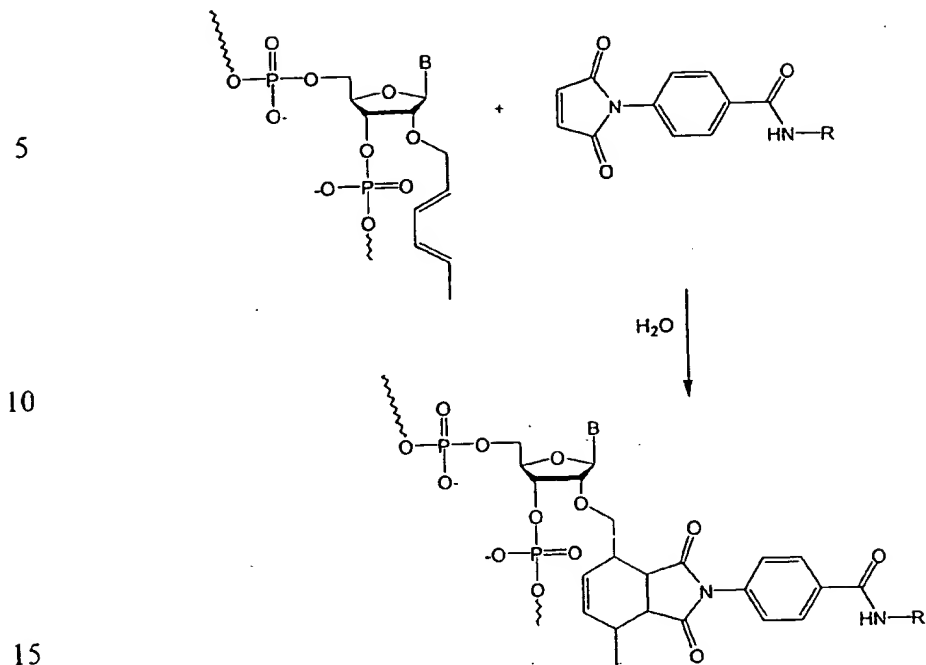
Example 4 demonstrates that the Diels-Alder cycloaddition reaction provides a powerful tool for chemoselective conjugation of a high molecular weight polyethylene glycol to an oligonucleotide in high yield. This Example describes the Diels-Alder reaction of a 28-mer oligonucleotide (oligonucleotide-5'-diene (12) bearing a 5'-terminal 3,5-hexadienephosphate with PEG5K-maleimide methylether and PEG20K-maleimide methylether.

Example 5 describes the dimerization of oligonucleotide-5'-diene (12) with 1,6 bismaleimidio hexane.

Example 6 describes the synthesis of 2'-(2,4-hexadienoxy)uridine-3'-thymidinephosphate (24). The preparation of 2'-O-hexadiene-nucleotides (Scheme 3) and all protected and phosphitylated derivatives thereof enables the diene moiety to be placed anywhere throughout an oligonucleotide or nucleotide analog, as well as, multiple times throughout the oligonucleotide. It is therefore possible to conjugate multiple detectors, peptides or sites of medicinal activity into one nucleotide by Diels-Alder cycloaddition.

30

SCHEME 3



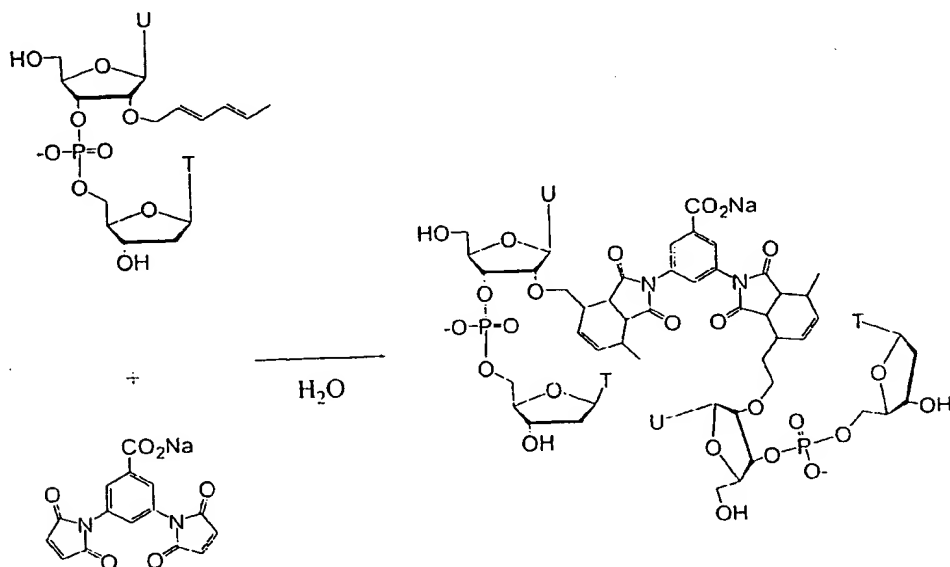
It is also possible to dimerize oligonucleotides integrated with 2'-O-hexadiene-bases as illustrated in Scheme 4. An illustrative dimerization reaction is described in Example 7.

SCHEME 4

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reactions occur in a concerted fashion, with Diels-Alder cycloadditions giving 6 member rings and 1,3-dipolar cycloadditions giving 5 member rings. Example 13 illustrates the 1,3-dipolar cycloaddition of 3'-azido-dideoxythymidine (AZT) with N-ethyl maleimide.

5 Bioconjugation with peptides and proteins is also possible provided that the peptide or protein can be derivatized with a moiety capable of undergoing a cycloaddition reaction. One approach to derivatizing peptides and proteins is to incorporate the reactive moiety into an amino acid, which can then be incorporated into the peptide or protein. Example 14 (Scheme 19) describes the synthesis of the
10 fully protected, diene modified amino acid alcohols (49) and (50). All of the bioconjugations described for oligonucleotides are applicable to peptides and proteins which have been functionalized with a moiety capable of undergoing a cycloaddition reaction. It is possible to incorporate numerous modified amino acids into a peptide for multiple sites of bioconjugation or multimerization. Similarly, other amino acids,
15 such as arginine may be modified for use as a 1,3-dipole for 1,3-dipolar cycloadditions.

The method of this invention can be extended to the preparation of heterodimers. Heterodimerization may be desirable to improve the pharmacokinetics or stability of a macromolecule. As illustrated in Example 15 (Schemes 20-22) a
20 diene functionalized macromolecule, such as an oligonucleotide, peptide or protein, may be conjugated to a dienophile functionalized macromolecule to produce the heterodimer. There may be more than one site of functionalization for either substrate, leading to crosslinking, more than one site of functionalization for only one substrate, giving a branched oligonucleotide, or single site functionalization for both
25 substrates, giving a heterodimer.

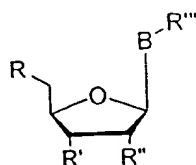
Example 16 (Scheme 24) illustrates the bioconjugation of a macromolecule using an ene cycloaddition reaction. In this example an oligonucleotide is used for purposes of illustration, however the reaction can be performed using any suitably labeled macromolecule. Example 16 also describes the preparation of an
30 oligonucleotide derivatized at the 5'-end with an ene moiety (Scheme 23). As in the

case of a diene moiety (discussed above), an ene moiety can be attached to any position on an oligonucleotide.

Example 17 illustrates the bioconjugation of a macromolecule using a [4+3] cycloaddition reaction. In this example an oligonucleotide is used for purposes of illustration, however the reaction can be performed using any suitably labeled macromolecule. Example 17 also describes the synthesis of an oligonucleotide derivatized with a furan.

In one embodiment the method of this invention can be used to synthesize prodrugs. Prodrugs are modified drugs adapted to release the drug after delivery. Often, prodrugs are linked to "carrier" molecules, and ideally, upon reaching their target, they are metabolized to a biologically active compound. Oligonucleotides are ideal carrier molecules in that they are easily conjugated and can be designed to bind to selected targets. Furthermore, large libraries of prodrug conjugated oligonucleotides may be easily synthesized by reacting a diene or dienophile modified nucleoside or nucleotide with a dienophile or diene modified prodrug or small molecule. An example of this is illustrated in Example 18 (Schemes 27 and 28).

The diene or dienophile may be placed anywhere on the sugar or base moiety of a free nucleoside or nucleotide monomer in any covalent manner as illustrated by the following structure:



More than one diene or dienophile may be incorporated into a single free nucleoside or nucleotide monomer. Additionally, more than one diene or dienophile modified monomer may be incorporated into an oligonucleotide. The incorporation of numerous prodrug or bioactive molecules could then be bioconjugated to a single nucleoside or nucleotide for more efficient delivery or improved pharmacokinetics.

The transcription of DNA templates catalyzed by a DNA dependent RNA polymerase, such as T7 RNA polymerase, is a prominent method for generation of RNA libraries and for preparation of active RNA molecules such as ribozymes. When such a transcription is initiated with a nucleoside or nucleotide bearing a diene or dienophile group a handle is introduced at the 5'-terminus which allows for subsequent selective conjugation of this RNA transcript to another macromolecule, a diagnostic detector, a crosslinking reagent, or a polymer or resin bearing one or several corresponding diene or dienophile groups. Example 19 describes the conjugation of such a transcript at a specific site on a wafer.

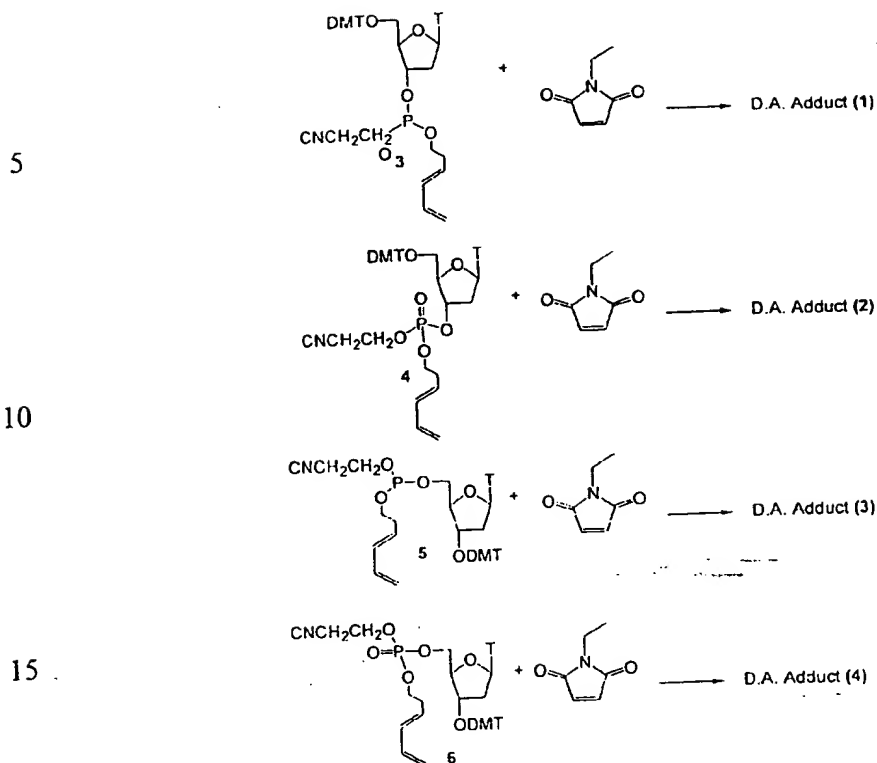
The following examples are presented for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLES

Example 1. Diels-Alder reactions of hexadiene phosphate nucleosides

The concept of addition of a diene phosphoramidite to an oligonucleotide for the purpose of subsequent conjugation to a dienophile bearing moiety was first tested in a model system (Scheme 4). The rate of cycloaddition of the 5'-protected thymidine 3'-(3,5-hexadiene)-(2-cyanoethyl)phosphite triester (3), the corresponding phosphate triester (4), the thymidine 5'-(3,5-hexadiene)-(2-cyanoethyl)phosphite triester (5), and the corresponding phosphate triester (6) with N-ethylmaleimide were compared. In most cases the Diels-Alder reaction was essentially complete within 10 hours at room temperature.

SCHEME 5



Preparation of the 5'-DMT-thymidine 3'-(3,5-hexadiene)-(2-cyanoethyl)phosphite triester (3). Compound 3 (Scheme 5) was prepared by reaction of (2-cyanoethyl)-N,N-diisopropyl-3,5-hexadiene phosphoramidite with 5'-O-DMT-thymidine. Briefly, in an argon purged, septum sealed 100 mL round bottom flask, equipped with a stir bar, was placed 5'-O-DMT-thymidine (2.16 g, 4 mmol). The flask was charged with 0.5 M tetrazole in acetonitrile (ACN) (40 mL, 5 equiv.) followed by (2-cyanoethyl)-N,N-diisopropyl-3,5-hexadiene phosphoramidite (4.45 mL, 1.5 equiv.). The reaction was allowed to stir for 45 minutes, during which time the solution turned from cloudy to clear. The mixture was poured into a 250 mL separatory funnel containing ethyl acetate (80 mL). The organic phase was washed twice with 40 mL of 2.5% sodium bicarbonate and once with brine. The organic phase was dried with MgSO_4 , the solvent was removed and the oil was chromatographed on silica (3:1 ethyl acetate/hexanes, column pre-treated with

diisopropyl ethyl amine). The resultant oil was foamed with acetone to yield 2.51 g (85%) of 95+% pure 3 by NMR.

Preparation of the 5'-DMT-thymidine 3'-(3,5-hexadiene)-(2-

- 5 cynoethyl)phosphate triester (4). Into a 100 mL round bottom flask was placed compound 3 (0.37 g, 0.5 mmol) and ACN (20 mL) with stirring. NaIO₄ (0.1 M in water, 2.5 equiv.) was added to the stirring mixture and allowed to react for 20 minutes. The mixture was then poured into 100 mL of EtOAc which was washed with water (2x40 mL) and brine (1x40 mL). The organic layer was dried with
- 10 MgSO₄, followed by filtration and solvent removal. The resultant oil was foamed with chloroform to give 0.3 g (80%) of pure 4 by NMR.

Preparation of the thymidine 5'-(3,5-hexadiene)-(2-cyanoethyl)phosphite

- 15 triester (5). Compound 5 (Scheme 4) was prepared by reaction of 5'-O-(2-cyanoethyl)-N,N-diisopropyl phosphoramidite thymidine 3'-O-DMT with 3,5-hexadien-1-ol. Briefly, in an argon purged, septum sealed 100 mL round bottom flask, equipped with a stir bar, was placed 5'-O-(2-cyanoethyl)-N,N-diisopropyl phosphoramidite thymidine 3'-O-DMT (0.5 g, 0.68 mmol). The flask was charged with 0.5 M tetrazole in ACN (7 mL, 5 equiv.) followed by 3,5-hexadien-1-ol (0.10 g,
- 20 1.5 equiv.). The reaction was allowed to stir for 45 minutes. The mixture was poured into a 250 mL separatory funnel containing ethyl acetate (80 mL). The organic phase was washed twice with 40 mL of 2.5% sodium bicarbonate and once with brine. The organic phase was dried with MgSO₄, the solvent was removed and the oil was chromatographed on silica (3:1 ethyl acetate/hexanes, column pre-treated with
- 25 diisopropyl ethyl amine). The resultant oil was foamed with acetone to yield 0.43 g (87%) of 95+% pure 5 by ¹H and ³¹P NMR.

Preparation of the thymidine 5'-(3,5-hexadiene)-(2-cyanoethyl)phosphate

- 30 triester (6). Into a 100 mL round bottom flask was placed compound 5 (0.22 g, 0.30 mmol) and ACN (10 mL) with stirring. Aqueous 0.1 M NaIO₄ (3 mL, 5 equiv.) was added to the stirring mixture and allowed to react for 20 minutes. The mixture was

then poured into 50 mL of ethyl acetate which was washed with water (2x40 mL) and brine (1x40 mL). The organic layer was dried with MgSO_4 , followed by filtration and solvent removal. The resultant oil was foamed with chloroform to give 0.21 g (94%) of pure **6** by NMR.

5

Measurement of Cycloaddition Reaction Rates. Approximately 0.01 mmol of the diene nucleoside and 1.25 equivalents of N-ethylmaleimide was weighed into an NMR tube. The reaction was dissolved in 0.5 mL of MeCN-d_3 and 0.5 mL of deuterium oxide. DMF-d_7 was added until all of the precipitate formed dissolved.

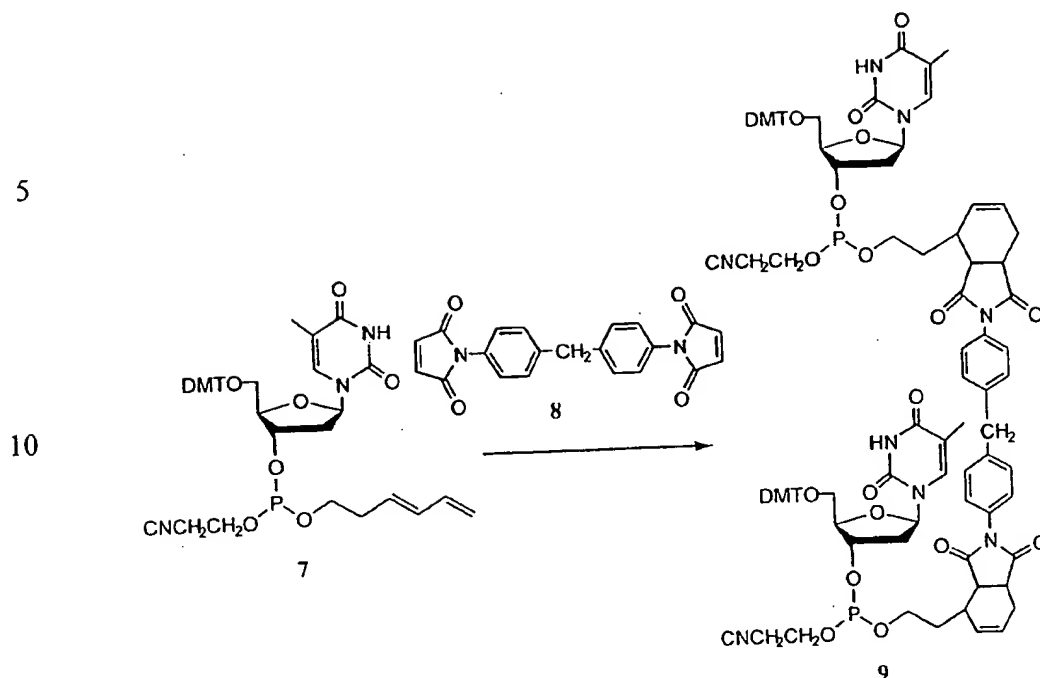
10 For NMR measurements, a drop of reaction mixture was removed, placed in another NMR tube, and diluted with an appropriate amount of MeCN-d_3 , which is used to lock the sample.

The calculations were made from integration of one aliphatic proton formed during the reaction ($\delta=5.7$) versus the disappearance of a diene proton ($\delta=6.1$). The calculation is the integration of $\delta=5.7$ divided by the sum of both integrations ($\delta=5.7$ and $\delta=6.1$) times 100%. The results of the rate studies are illustrated graphically in Figures 1 and 2.

15

Example 2. Dimerization of 5'-DMT-thymidine 3'-hexadiene-(2-cyanoethyl)phosphite
20 with 4,4'-dimalcimido-diphenylmethane

SCHEME 6



20 The dimerization of 5'-DMT-thymidine 3'-hexadiene-(2-cyanoethyl)phosphite (7) with 4,4'-dimaleimidodiphenylmethane dimaleimide (8) is illustrated in Scheme 6. The concentration of 5'-DMT-thymidine 3'-hexadiene-(2-cyanoethyl)phosphite was kept at 0.36 M and the concentration of dimaleimide at 1 equivalent. There were few side products. The mixture was sampled intermittently over 22 hours. Figure 3 illustrates graphically the amount of mono-Diels-Alder cycloaddition product (mono conjugate) and dimer conjugate present (9) in the reaction mixture over the 22 hour period of time.

25 Example 3. Preparation of derivatized phosphoramidites

Synthesis of 2-cyanoethyl-(3,5-hexadiene) phosphoramidite (11A).

30 Into an argon purged, septum sealed round bottom flask was placed 3,5-hexadien-1-ol (2.43 g, 24.8 mmol). The flask was charged with methylene chloride (50 mL) and diisopropylethyl amine (14.5 g, 5.0 equiv.) with stirring. The flask was then charged with 2-cyanoethyl-N,N'-diisopropylchlorophosphoramidite (5.00 g, 22.5

mmol) and stirred for 45 minutes. The reaction mixture was diluted to 100 mL with methylene chloride and then washed with 2.5% NaHCO₃ (2x50 mL) and brine (1x50 mL). The organic phase was dried with MgSO₄, the solvent removed and the oil dried under high vacuum for 18 hours to yield compound **11A** in 99+% purity by ¹H NMR and ³¹P NMR.

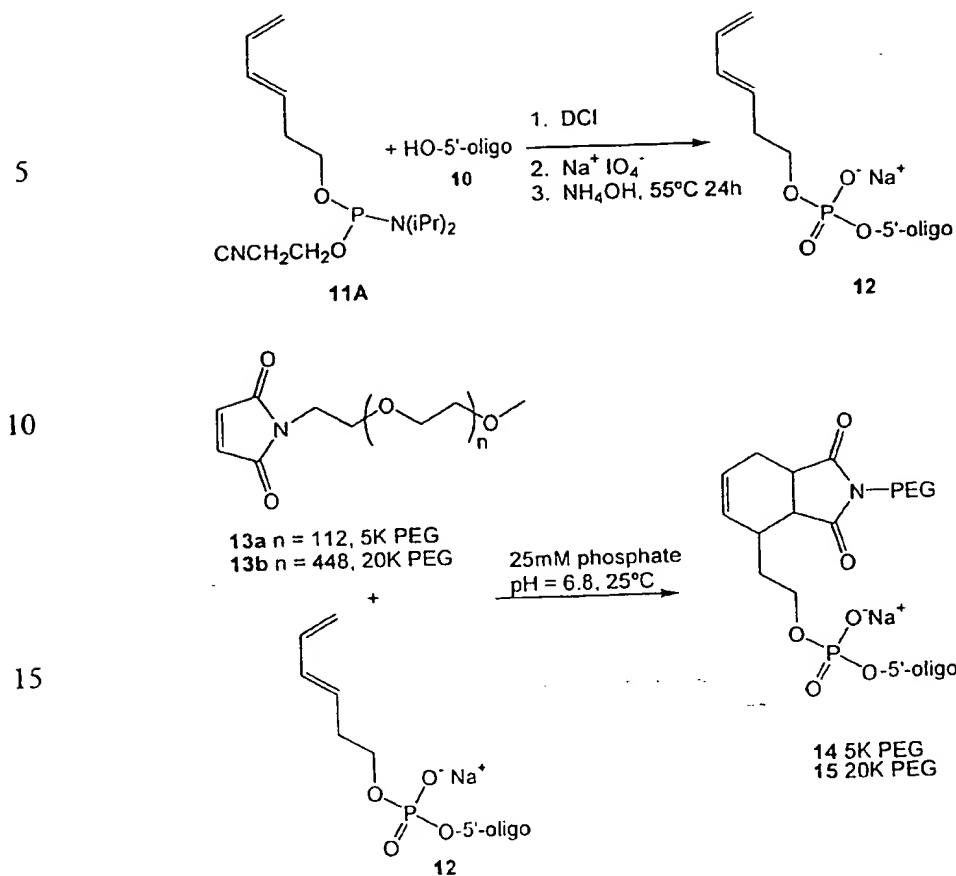
Synthesis of bis(3,5-hexadiene)-N,N'-diisopropyl phosphoramidite (**11B**).

Into an argon purged, septum sealed round bottom flask was placed 3,5-hexadien-1-ol (2.56 g, 26.1 mmol). The flask was charged with methylene chloride (50 mL) and diisopropylethyl amine (16.0 mL, 5.0 equiv.) with stirring. The flask was then charged with N,N'-diisopropyl phosphoramidous dichloride (2.5 g, 12.4 mmol) and allowed to stir for 45 minutes. The reaction mixture was diluted to 100 mL with methylene chloride then washed with 2.5% NaHCO₃ (2x50 mL) and brine (1x50 mL). The organic phase was dried with MgSO₄, the solvent removed and the oil dried under high vacuum for 18 hours to yield compound **11B** in 99+% purity by ¹H NMR and ³¹P NMR.

Example 4. Conjugation of PEG-maleimide methylether to a 28-mer oligonucleotide bearing a 5'-terminal 3,5-hexadienephosphate

40

SCHEME 7



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Oligonucleotide Synthesis. A 28-mer DNA having the sequence 5'-HO-CCAGTACAAGGTGCTAAACGTAATGG[3'3'T]T-3' (**10**) was prepared on a Milligen 8800 oligonucleotide synthesizer using standard solid phase protocols at the 300 μmole scale. After completion of the 5' terminal solid phase addition cycle, the

25 3.5 hexadiene-1-ol phosphoramidite (**11A**) was added (0.2M acetonitrile solution, 4 equivalents dicyanoimidazole (DCI), 30 minutes coupling time) to form the corresponding phosphite triester (Scheme 7). The phosphite triester was then oxidized with 0.5M NaIO_4 in water for 10 minutes to form the desired phosphate triester. The excess oxidant was removed by washing with water followed by acetonitrile. Use of

30 the standard iodine oxidation protocol resulted in destruction of the diene moiety. The oligonucleotide was cleaved from the solid support and deprotected under standard

conditions. Anion exchange analysis of the crude deprotected oligonucleotide showed 45% full length oligonucleotide-5'-diene.

The crude oligonucleotide-5'-diene was purified by reverse phase HPLC on a Hamilton PRP-1 column using a tetrabutylammonium bromide/acetonitrile gradient.

- 5 The purified oligonucleotide was pooled and the tetrabutylammonium salt exchanged for sodium on the PRP-1 column, the excess sodium was removed with a water wash and the sodium salt of the oligonucleotide was eluted with approximately 50% acetonitrile. The purified oligonucleotide (12) was then lyophilized to a white powder. This material was 90% pure by anion exchange HPLC (Dionex Nucleopak
10 strong anion exchange column, Tris/sodium chloride gradient at 85°C). This material was analyzed by mass spectroscopy (electrospray), expected mass = 8796; observed mass = 8796.

- 5K Polyethylene glycol conjugation. The lyophilized oligonucleotide-5'-diene (12)
15 was dissolved in 25 mM phosphate (pH=6.8, 8 μ M, approximately 74mg/mL). To this solution was added two equivalents of monomethoxy-PEG-maleimide (13a) (MW = 5,000, Shearwater Polymers) (Scheme 7). After 18 hours at 25°C all of the diene labeled oligonucleotide had undergone coupling with the maleimide-PEG to give the 5K PEG Diels-Alder product (14). The product was isolated by reverse phase
20 chromatography. (See Figures 4 and 5).

- A similarly prepared oligonucleotide sample that lacked the 5' diene label did not couple to the maleimide PEG. Addition of acetonitrile to the Diels-Alder reaction slowed down the conjugation rates. The addition of 10% acetonitrile slowed the rate by nearly half the value in water alone. These oligo-5'-dienes thus behave like small
25 non charged dienes in their reactivity in aqueous solution.

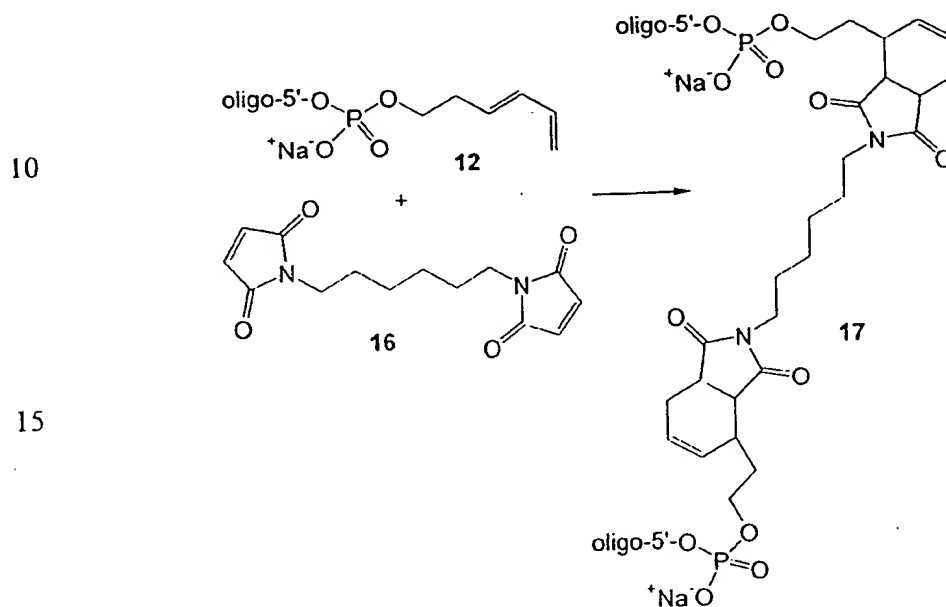
- 20K Polyethylene glycol conjugation. The lyophilized oligonucleotide-5'-diene (12) was dissolved in 25 mM phosphate (pH=6.8, 5 μ M, approximately 55 mg/mL). To this solution was added two equivalents of monomethoxy-PEG-maleimide (13b)
30 (MW = 20,000, Shearwater Polymers) (Scheme 7). After 18 hours at 25°C all of the diene labeled oligonucleotide had undergone coupling with the maleimide-PEG to

give the 20K PEG Diels-Alder product (15). The product was isolated by reverse phase chromatography.

Example 5. Conjugation of oligonucleotide-5'-diene (12) with 1,6 bismaleimidio

5 hexane

SCHEME 8



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A solution of oligonucleotide (12) was treated with 1/3 an equivalent of 1,6-bismaleimidio hexane (16) (Pierce) at 25°C (Scheme 8). After 16 hours the dimer conjugate (17), had formed in approximately 80% yield (based on maleimide), very little mono conjugate was obtained. The dimer product was isolated by anion exchange chromatography and analyzed by mass spectroscopy (electrospray).
 25 expected mass = 17872 observed mass = 17873.

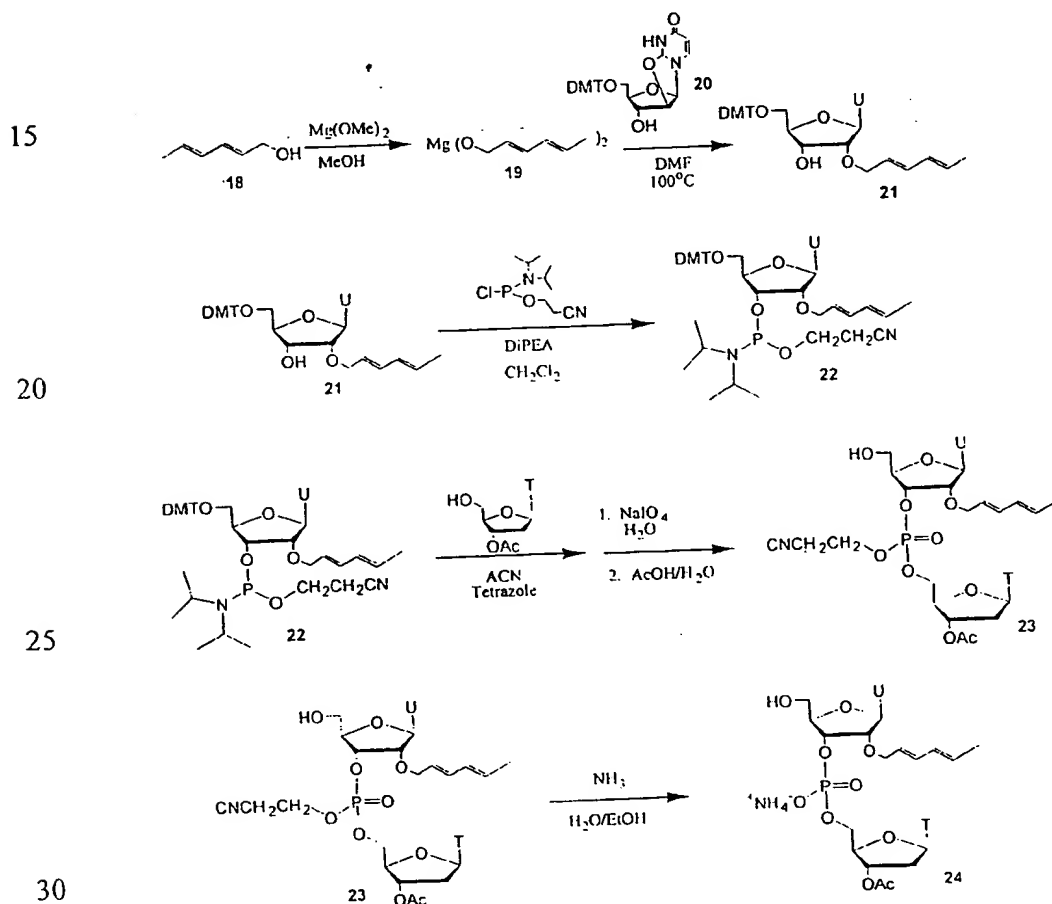
Diels-Alder adducts have also been prepared with N-ethyl maleimide. Briefly, approximately two equivalents of N-ethyl maleimide was added to an aqueous solution of oligonucleotide-5'-diene (12) at 25°C. After four hours the reaction was
 30 complete. The products were isolated by anion exchange chromatography and

analyzed by mass spectroscopy (electrospray), expected mass = 8921 observed mass = 8922.

Example 6. Preparation of a 2'-O-(2,4-hexadiene)uridine-3'-O-thymidinephosphate

- DMT-anhydrouridine is known to undergo reaction with $Mg(R)_2$ (where R= alkoxy) to yield 2'-substituted nucleosides. As demonstrated below, this chemistry can be used to prepare nucleosides substituted at the 2'-position with a diene. The 2'-substituted nucleoside can then be incorporated into a nucleotide or nucleotide analog. This is demonstrated by the solution phase synthesis of 2'-O-(2,4-hexadiene)uridine-3'-O-(5'-O-acetylthymidine) phosphate (24) (Scheme 9).

SCHEME 9



mL, 7 equiv.) and cyanoethyl-N,N'-diisopropylchlorophosphoramidite (2.14 mL, 2 equiv.). The reaction mixture was stirred for 45 minutes. The mixture was then washed with aqueous 2.5% NaHCO₃ (2x50 mL) and brine (1x50 mL), dried with MgSO₄, filtered and the solvent was removed *in vacuo*. The yellow oil was chromatographed with silica and 1:1 EtOAc/hexanes to afford 3.14 g (80%) of 5'-O-DMT-2'-O-(2,4-hexadiene)uridine-3'-O-cyanoethyl-N,N'-diisopropylphosphoramidite (22) that was pure by ¹H NMR.

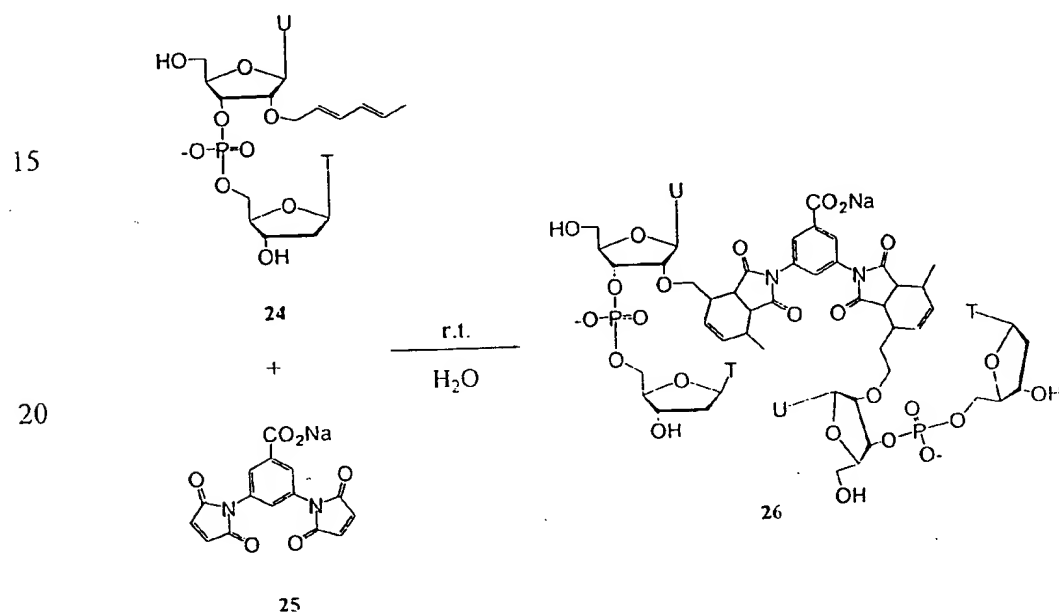
Preparation of 2'-O-(2,4-hexadiene)uridine-3'-O-(5'-O-acetylthymidine) cyanoethyl phosphate (23). 5'-O-DMT-2'-O-(2,4-hexadiene)uridine-3'-O-cyanoethyl-N,N'-diisopropyl phosphoramidite (22) (2.18 g, 2.64 mmol) and 3'-O-acetylthymidine (0.7523 g, 2.65 mmol) were placed in a 250 mL round bottom flask equipped with a stir bar. The flask was charged with 0.5 M tetrazole in ACN (25 mL, 4.5 equiv.) with stirring. The reaction was stirred for 25 minutes then washed with aqueous 2.5% NaHCO₃ (2x50 mL) and brine (1x50 mL). The resulting wet oil was taken up in ACN (30 mL) and treated with aqueous 0.5 M NaIO₄ (13 mL, 2.5 equiv.). The mixture was stirred for 10 minutes, then poured into EtOAc (100 mL) before washing with water (2x50 mL) and brine (1x50 mL). The organic phase was dried with MgSO₄, filtered and the solvent was removed to afford a white solid. The solid was dissolved in AcOH/H₂O (4:1) and stirred for 1 hour. The acid and water was removed *in vacuo*. The oil was dissolved in MeCl₂ and precipitated with ether. The solid was then filtered and collected. The precipitation was performed 2 additional times on the supernatant. The solid was purified on silica (7% MeOH in MeCl₂) to afford 1.56 g (81.9%) of 2'-O-(2,4-hexadiene)uridine-3'-O-(5'-O-acetylthymidine) cyanoethylphosphate (23) that was pure by ¹H NMR.

Preparation of 2'-O-(2,4-hexadiene)uridine-3'-O-(5'-O-acetylthymidine) phosphate (24). 2'-O-(2,4-hexadiene)uridine-3'-O-(5'-O-acetylthymidine) cyanoethylphosphate (23) (1.36 g, 1.89 mmol) was dissolved with gentle heating in EtOH (40 mL). The solution was transferred to a 250 mL Pyrex® screw cap bottle with a Teflon® lined cap.

Concentrated ammonium hydroxide (150 mL) was added, the cap replaced and the bottle was vigorously shook and the initial pressure relieved. The bottle was then placed in a incubated shaker at 37°C after re-tightening the cap. The mixture was allowed to shake for 4.5 hours. The solvent was removed *in vacuo*, the foam was redissolved in H₂O/MeOH (1:1) and precipitated by ACN at -20°C overnight, yielding 1.08 g of white crystalline 2'-O-(2,4-hexadiene)uridine-3'-O-(5'-O-acetylthymidine) phosphate (24) that was pure by ¹H NMR with no acetamide contamination.

Example 7. Dimerization of 2'-O-(2,4-hexadiene)uridine-3'-thymidine phosphate with sodium 3,5-bismaleimidobenzoate

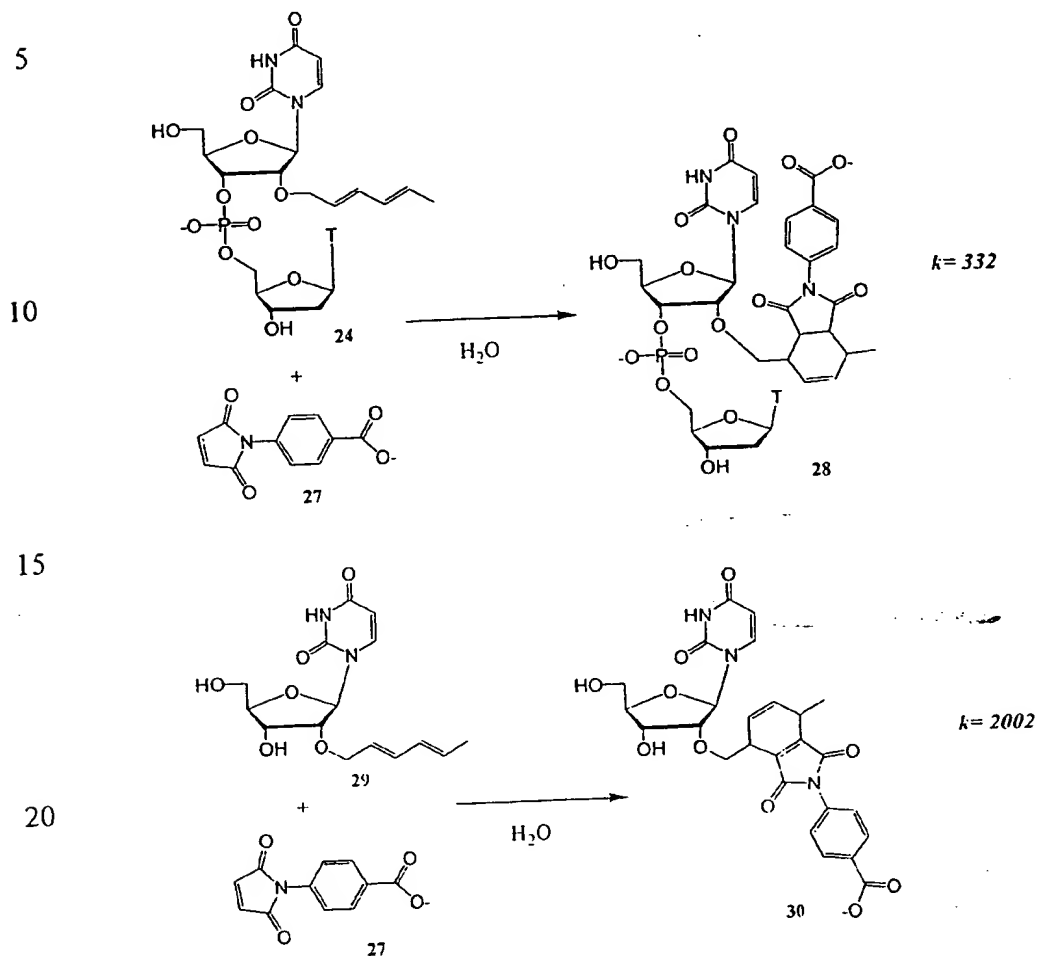
SCHEME 10



2'-O-(2,4-hexadiene)uridine-3'-thymidine phosphate (24) (20.1 mg, 0.031 mmol) was placed into a screw cap vial with sodium (3,5-bismaleimidobenzoate (25) (4.9 mg, 0.5 equiv.) and the mixture was dissolved in 1 mL of D₂O (Scheme 10). ¹H NMR and HPLC analysis (run as described in Example 8) show complete conversion to dimerized 2'-O-(2,4-hexadiene)uridine-3'-thymidine phosphate (26).

Example 8. Diels-Alder Reactions of 2'-O-(2,4-hexadiene)uridine-3'-thymidine phosphate and sodium 4-maleimidobenzoate

SCHEME 11



2'-O-(2,4-hexadiene)uridine-3'-thymidine phosphate (24) (21.5 mg, 0.0334 mmol) was weighed into a screw cap vial with sodium 4-maleimidobenzoate (27) (82.5 mg, 10 equiv.) and dissolved in 1 mL of D_2O . 200 μL was sampled periodically over 30 minutes and diluted to 600 μL with acetonitrile- d_3 . The reaction was kept at 0°C until analysis via HPLC or 1H NMR. HPLC analysis was performed on a BioCad 60 instrument fitted with an analytical Waters DeltaPak C-18 column using a 0-15%

acetonitrile gradient in 100 mM triethylamine/acetic acid buffer (pH = 7) run over 6 column volumes.

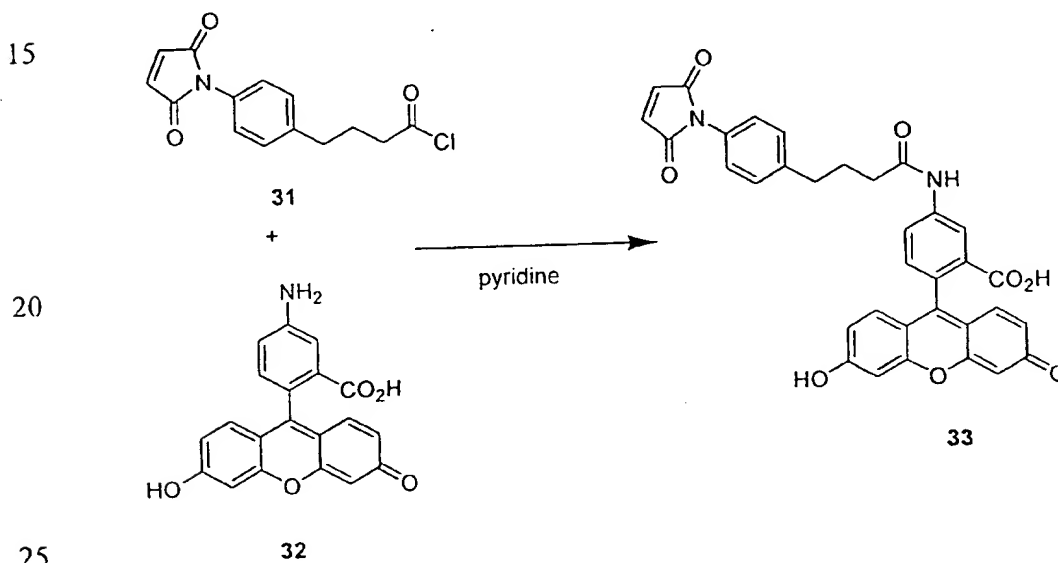
The reaction 2'-O-(2,4-hexadiene)uridine (29) (monomer) with sodium 4-maleimidobenzoate (27) was performed using the same reaction conditions to yield the Diels-Alder cycloaddition product (30).

Example 9. Conjugation of a fluorescent detector to a 2'-hexadiene bearing dimer oligonucleotide

Example 9 (Schemes 12-15) illustrates the conjugation of oligonucleotide-5'-dienes with various fluorescent detectors.

Preparation of 4'-maleimidophenylbutyramide conjugated fluorescein (33).

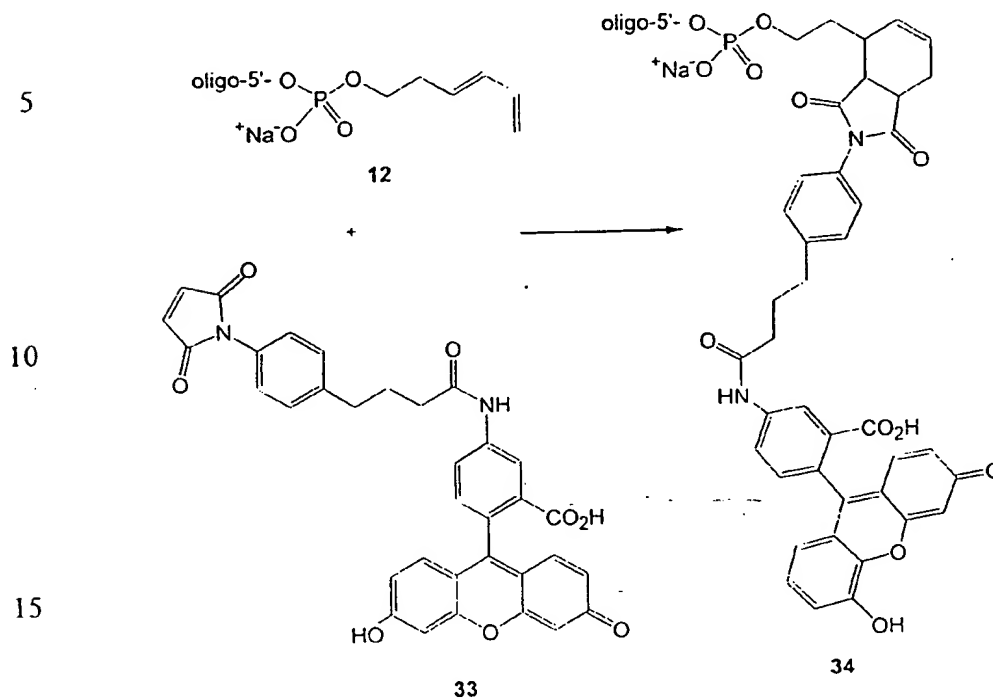
SCHEME 12



Fluorescein is commonly used as a fluorescent label for biologically interesting compounds. Fluorescamine (32) (Scheme 12), a commercially available amine derivatized fluorescein (a deep burgundy solid), was attached to maleimidophenylbutanoic acid. Amidolysis was achieved via the acid chloride of the maleimide-acid (31). The reaction was smooth followed by a relatively easy separation via column chromatography to yield a bright orange solid (33).

Diels-Alder reaction of oligonucleotide-5'-diene (12) with compound (33).

SCHEME 13

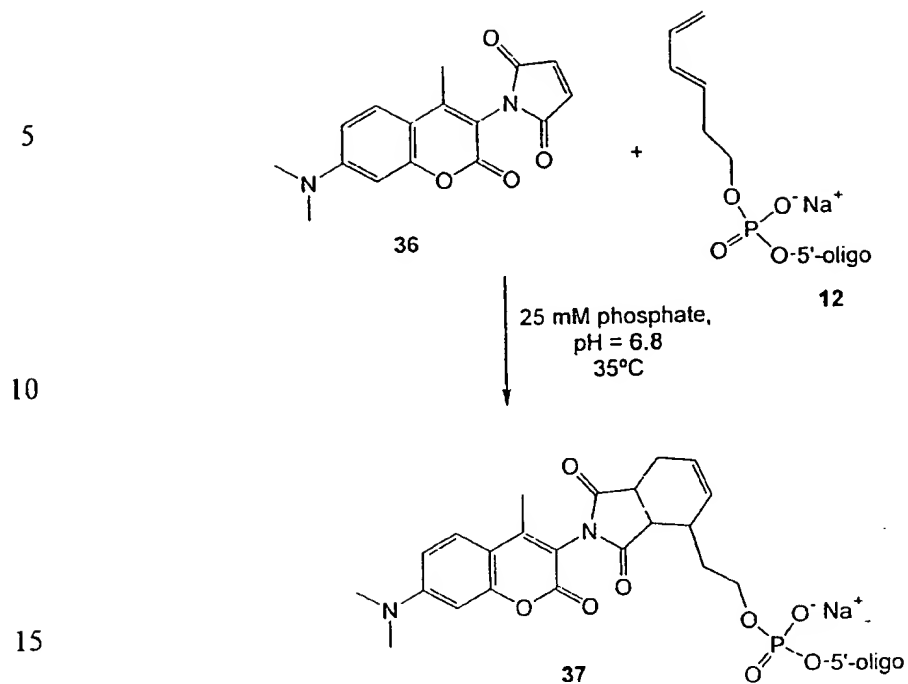


Approximately two equivalents of compound (33) were added to an aqueous solution of the oligonucleotide-5'-diene (12) at 25°C. After 20 hours the reaction was complete. The product (34) was isolated by reverse phase chromatography.

Diels-Alder reaction of 2'-O-(2,4-hexadiene)uridine-3'-O-(5'-O-acetylthymidine) phosphate (24) with compound (33).

51

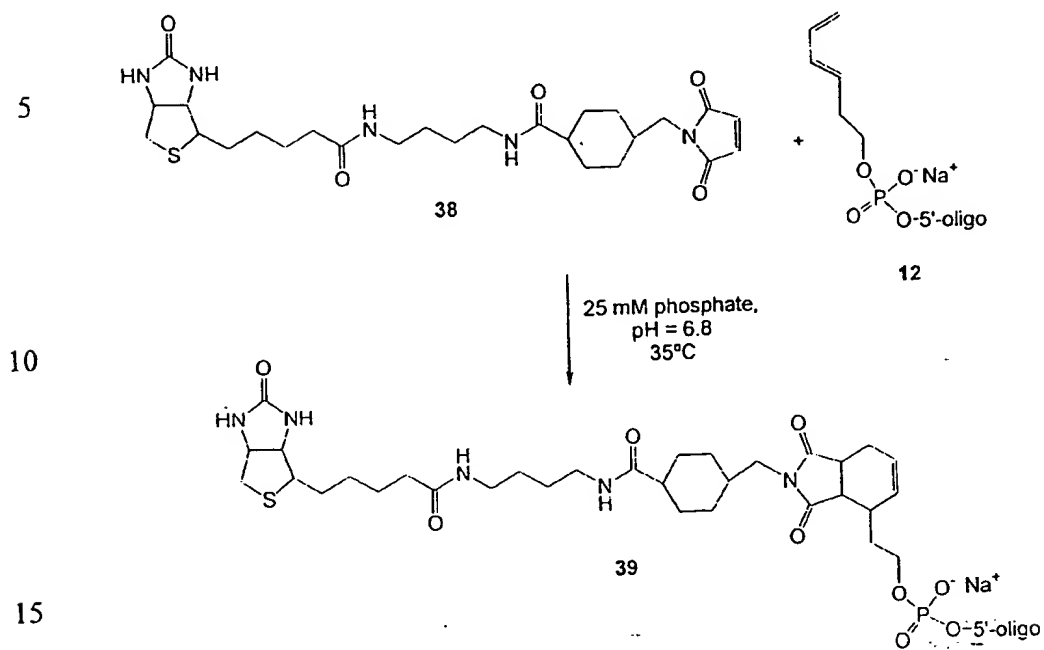
SCHEME 15



Approximately 1.3 equivalents of compound (36) a coumarin maleimide derivative (Aldrich Chemical) was added to an aqueous solution of the oligonucleotide-5'-diene (12) at 35°C. After 60 hours the reaction was complete. The product (37) was isolated by anion exchange chromatography (in approximately 90% yield) and analyzed by mass spectroscopy (electrospray), expected mass = 9096 observed mass = 9097.

Example 10. Conjugation of a biotin maleimide to oligonucleotide-5'-diene (12)

SCHEME 16



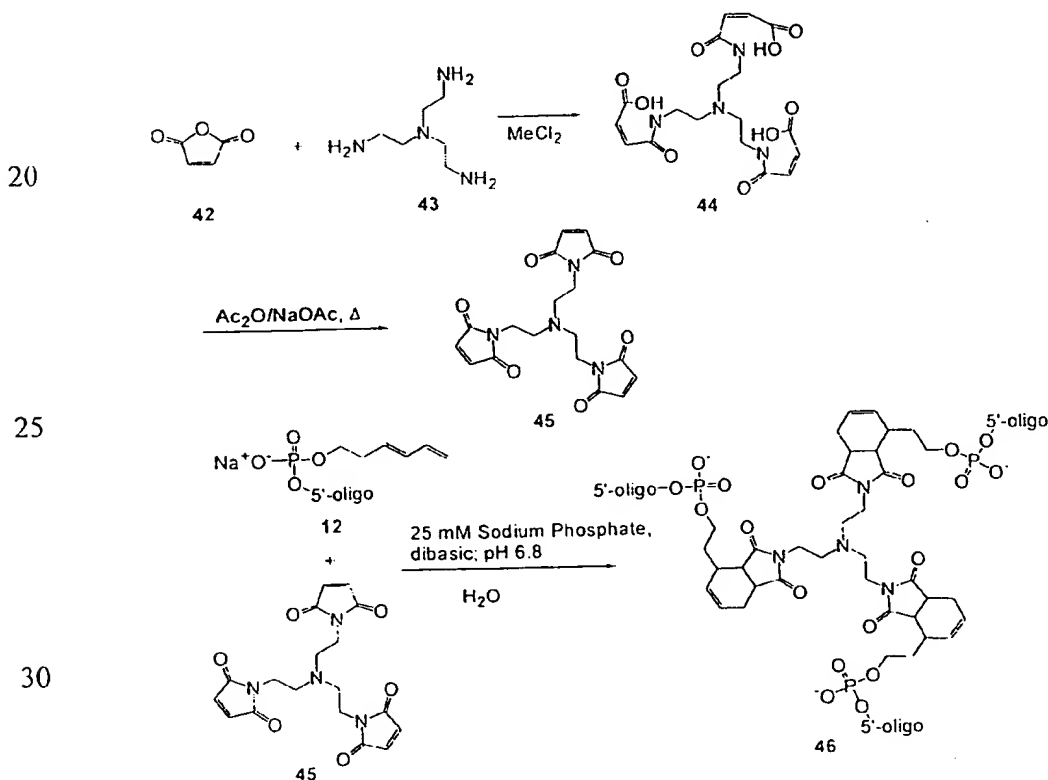
Approximately 2 equivalents of biotin maleimide (38) were added to an aqueous solution of the oligonucleotide-5'-diene (12) at 35°C. After 18 hours the reaction was complete. The product (39) was isolated by anion exchange chromatography (in approximately 90% yield) and analyzed by mass spectroscopy (electrospray), expected mass = 9332 observed mass = 9333.

Example 11. Conjugation of a dienophile bearing oligonucleotide to a diene bearing lipid for liposomal anchoring

A phospholipid bearing a diene unit can be used to anchor an oligonucleotide in the lipid bilayer of a liposome. A lipid such as linoleic acid is isomerized to the corresponding conjugated diene derivative under acidic conditions. This derivative is subsequently converted to the corresponding phospholipid. The phospholipid is poised to react with dienophiles in an aqueous environment to form adducts by cycloaddition. Due to the nature of the phospholipid orientation in a liposome, the adduct is located in the lipid bilayer (see United States Application No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Ligand Complexes" which is incorporated herein by reference in its entirety). Treatment of a maleimide derivatized oligonucleotide with a diene containing phospholipid would lead to the formation of a lipid oligonucleotide conjugate as formed by the Diels-Alder reaction.

Example 12. Multimerization of oligonucleotides

SCHEME 17



Synthesis of tris (2-maleimidoethyl) amine (TREM).

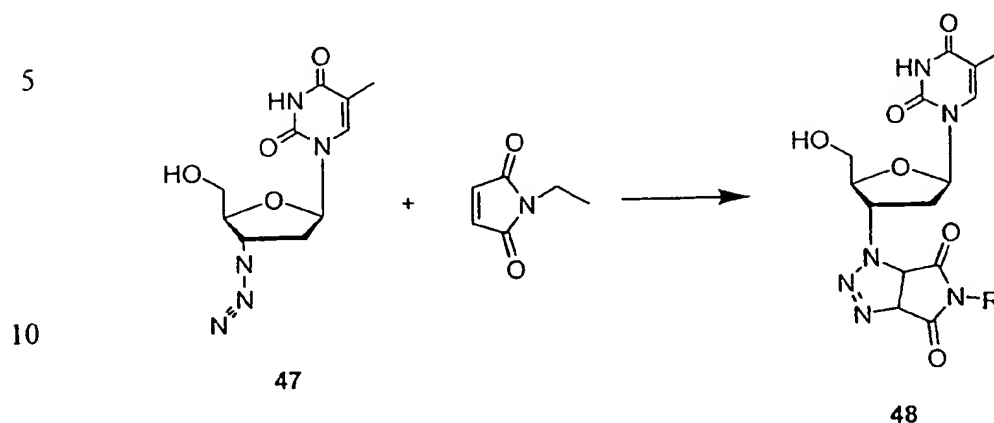
Maleic anhydride (42, 5.00 g, 50.9 mmol) was added to a solution of tris (2-aminoethyl) amine (43, 2.02 mL, 0.80 equiv.) in MeCl_2 (50 mL). After 2 minutes, a white solid began to precipitate out and the reaction was allowed to stir for an additional 2 hours at room temperature. The white solid was filtered off and NMR revealed the tri-maleamic acid adduct of TREN (44). Ring closure was performed using NaOAc in Ac_2O at 100°C for 3 hours. The reaction mixture was quenched with water, the acetic acid removed *in vacuo* and the product dried at high vacuum. The major product was then purified on a Biotage Flash 40 (5% ethyl acetate in hexanes) to afford a yellow solid that gave a ^1H NMR consistent with tris-(2-maleimidoethyl) ammonium acetate (45, TREM).

Synthesis of oligonucleotide trimer (46).

Stock solutions of 5'-hexadiene phosphate 12 (0.0075 M in Na_2PO_4 , pH 6.8) and TREM (0.011 M in acetone) were prepared. The 5'-hexadiene phosphate 12 (200 μL , 1.5 μmol) was added to a screw cap vial with TREM (45, 22.3 μL , 0.3 μmol). The reaction mixture was allowed to shake for 24 hours at which time a sample was taken. The sample was analyzed by HPLC (Dionex DNA column, 3.1 mL volume; 36-78% Buffer B over 16 column volumes; Buffer A: 25 mM Trizma, 1mM EDTA, 10% ACN in H_2O ; Buffer B: Same as Buffer A plus 1 M NaCl). The fractions of trimer (46) were collected and characterized via mass spectroscopy with an expected mass of 26777 and a measured mass of 26793, well within expected error of $\pm 3\%$.

Example 13. Bioconjugation via 1,3-cycloaddition

SCHEME 18



Into a 3 mL vial is weighed AZT (47) (10 mg, 37.4 μ mol) which is then dissolved in 0.8 mL of 0.05 M Na_3HPO_4 (pH 12). N-Ethyl maleimide ((9.4 mg, 75 μ mol) is weighed into a separate vial and dissolved in 1 mL of ethanol. Into the vial containing the AZT is placed 0.2 mL of the N-ethyl maleimide solution. The vial is capped, shaken well for 5 minutes and then allowed to react at 45°C for 3 hours to yield compound (48). A 100 μ L aliquot of the reaction mixture is diluted to 700 μ L, mixed well and analyzed via HPLC (Jupiter C-18 column, 2-40% acetonitrile in 100 mM triethylamine/acetic acid pH 7 buffer over 7 column volumes).

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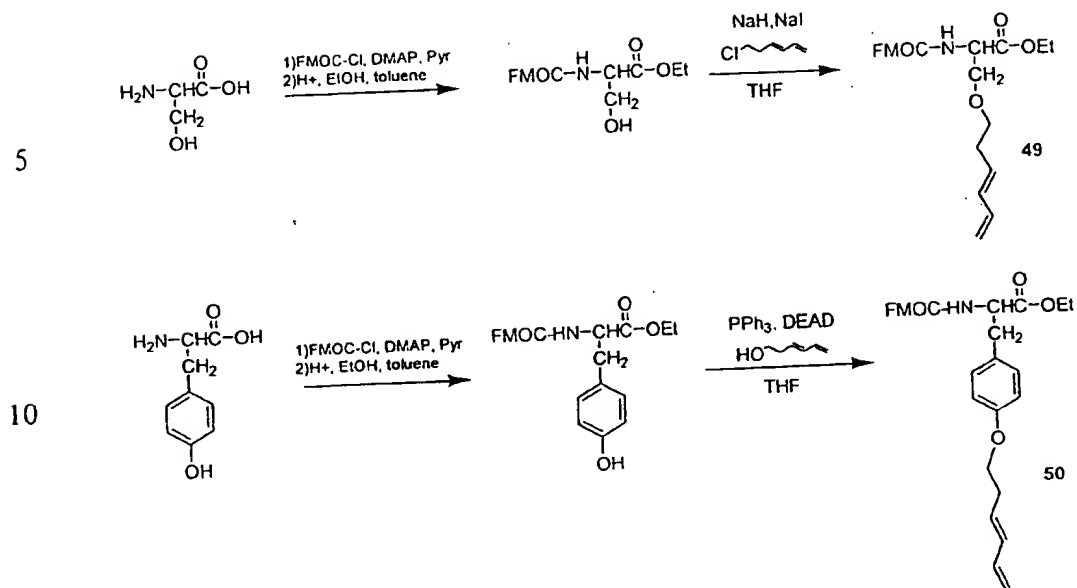
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Example 14. Preparation of diene modified amino acids

Scheme 19 illustrates the synthesis of the fully protected, diene modified amino acids (49) and (50).

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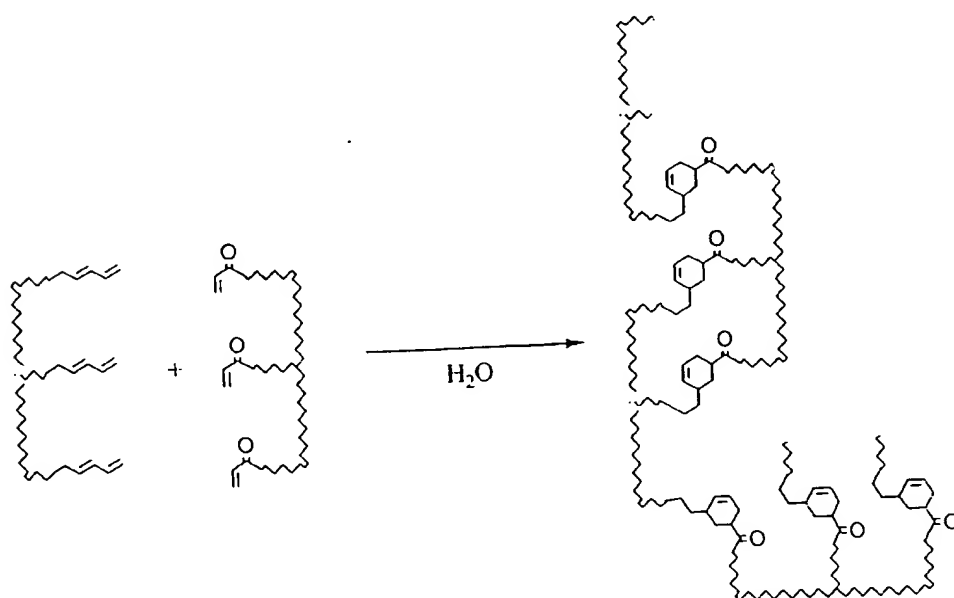
SCHEME 19



Example 15. Formation of "heterodimers" of oligonucleotides

Scheme 20 illustrates the heterodimerization of two macromolecules each of which has more than one site of functionalization leading to a crosslinked product.

SCHEME 20



Scheme 21 illustrates the heterodimerization of two oligonucleotides, wherein the first oligonucleotide has more than one site of functionalization and the second oligonucleotide has only a single site of functionalization giving a branched oligonucleotide product.

SCHEME 21

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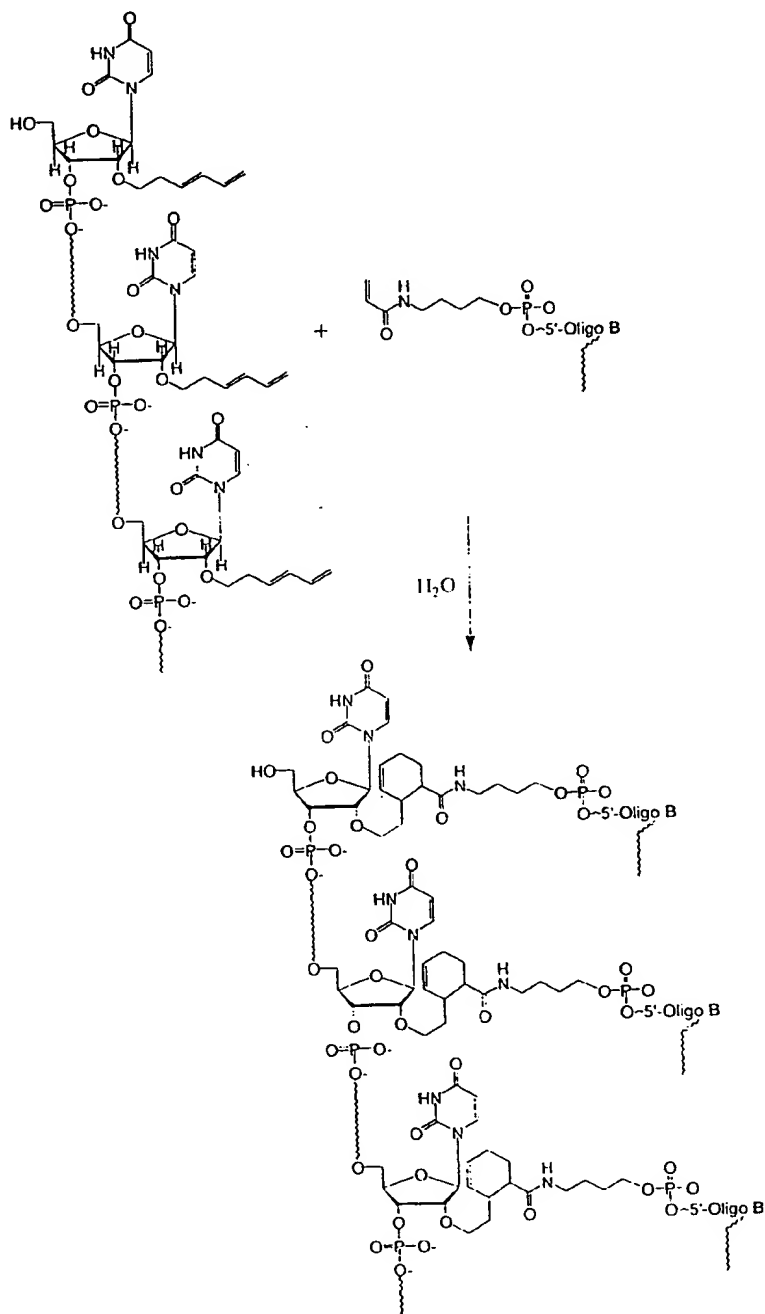
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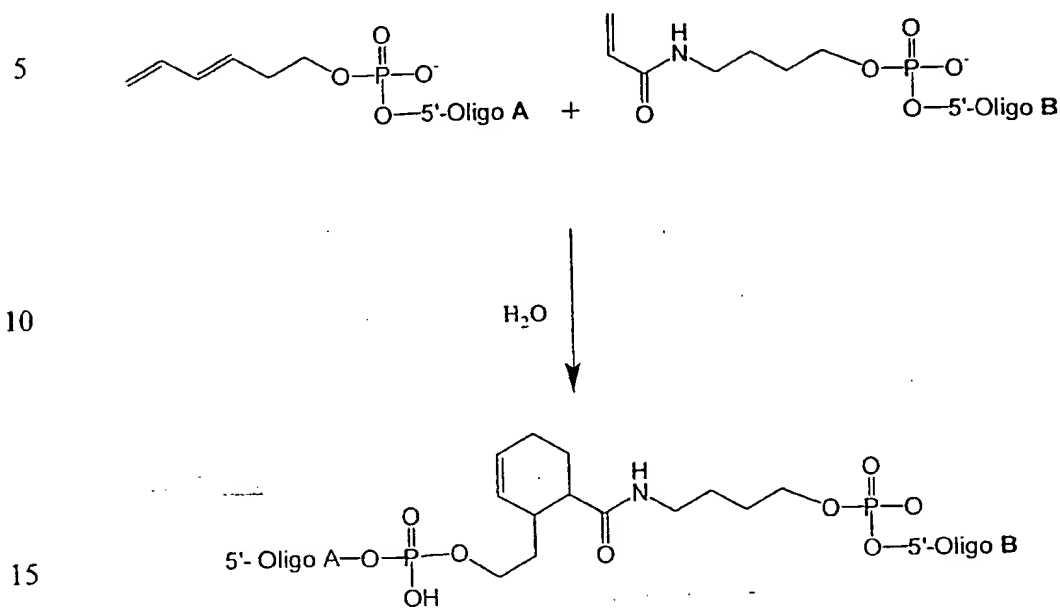
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Scheme 22 illustrates the heterodimerization of two oligonucleotides, each of which has only one site of functionalization.

SCHEME 22

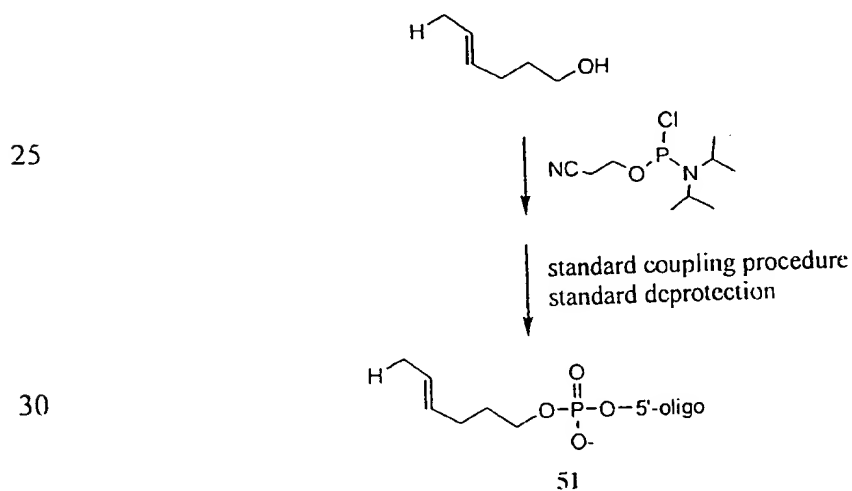


Example 16. Bioconjugation via an ene reaction

Synthesis of a 5'-ene phosphoramidite. An ene oligonucleotide is prepared as set

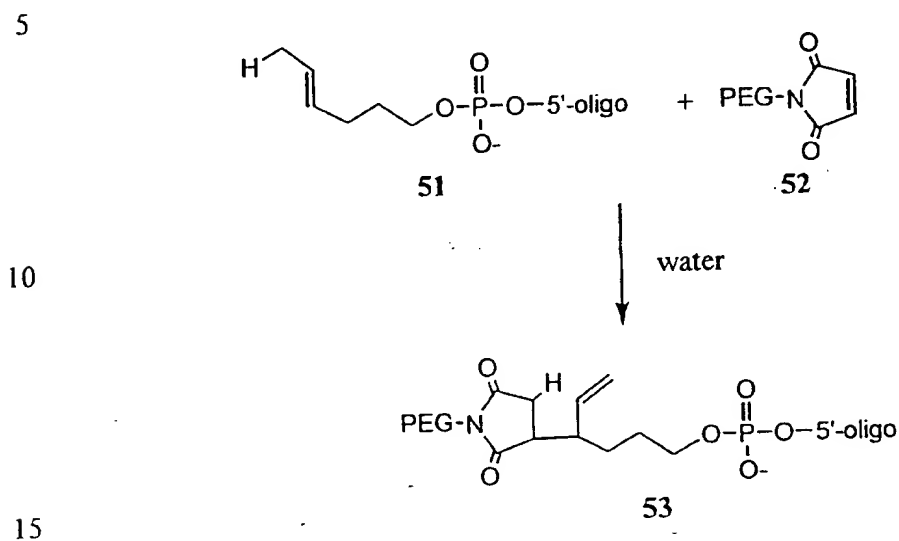
20 forth in Scheme 23.

SCHEME 23



Ene cycloaddition reaction. Treatment of ene derivatized oligonucleotide (51) with PEG-maleimide (52) at room temperature in water gives the bioconjugated oligonucleotide (53).

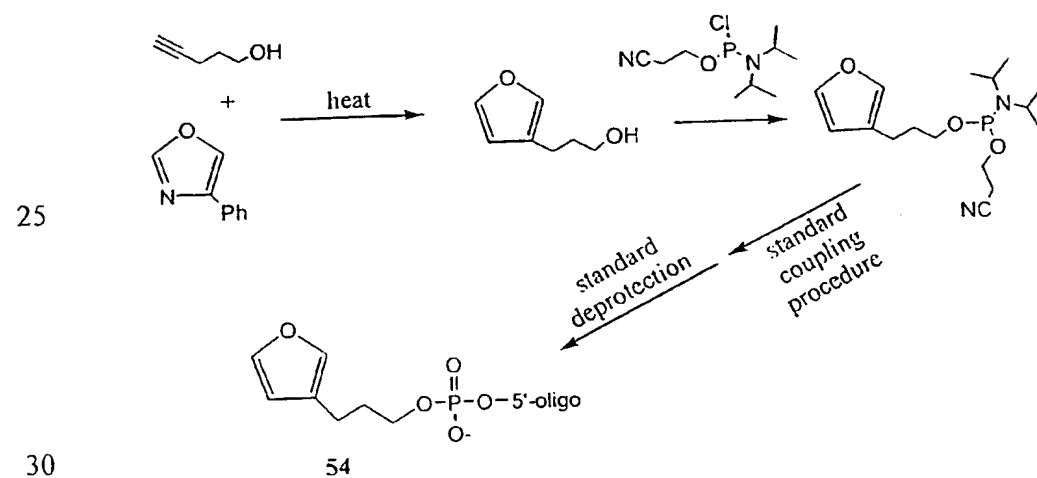
SCHEME 24



Example 17. Bioconjugation via [4+3] cycloaddition

Preparation of a furan derivatized oligonucleotide. A furan derivatized oligonucleotide is prepared as set forth in Scheme 25.

SCHEME 25



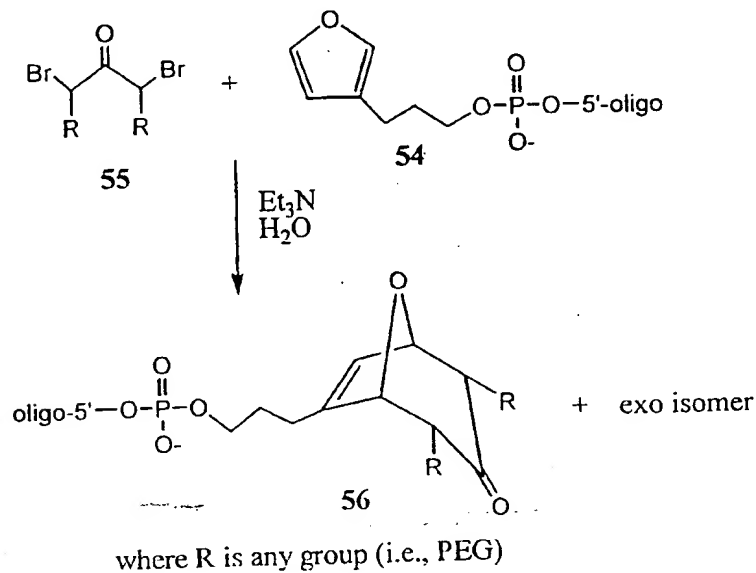
[4+3] Cycloaddition reaction. Treatment of furan derivatized oligonucleotide (54) with compound (55) at room temperature in water and triethylamine gives the derivatized oligonucleotide (56). (Lubineau (1997) *Tetrahedron Lett.* **38**:8031).

SCHEME 26

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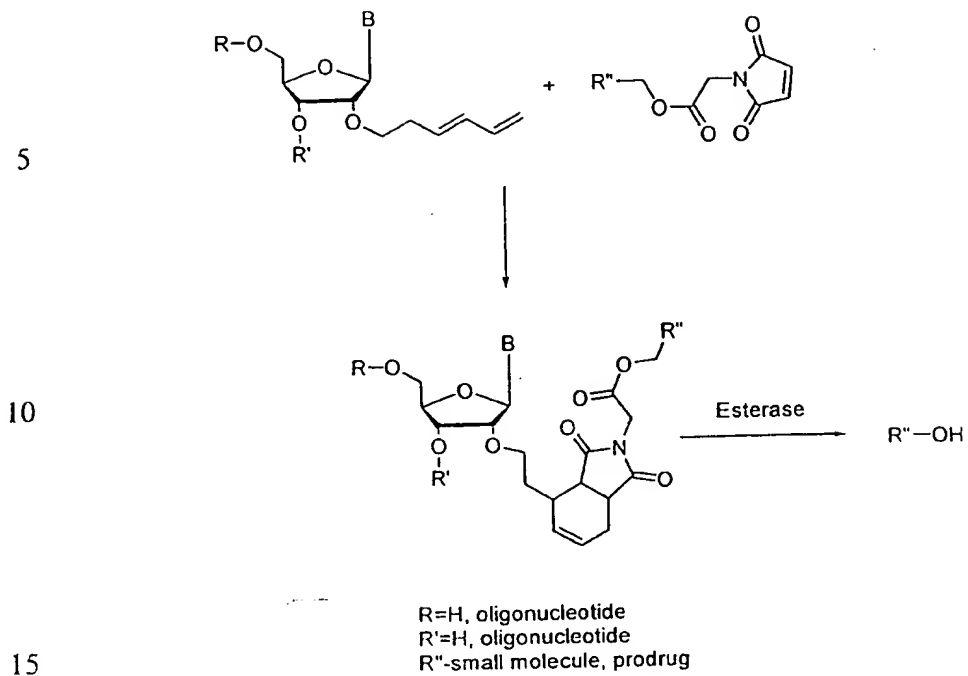
Example 18. Application of bioconjugation to the production of prodrug libraries

Schemes 27 and 28 illustrate the application of the method of this invention to the production of prodrug libraries.

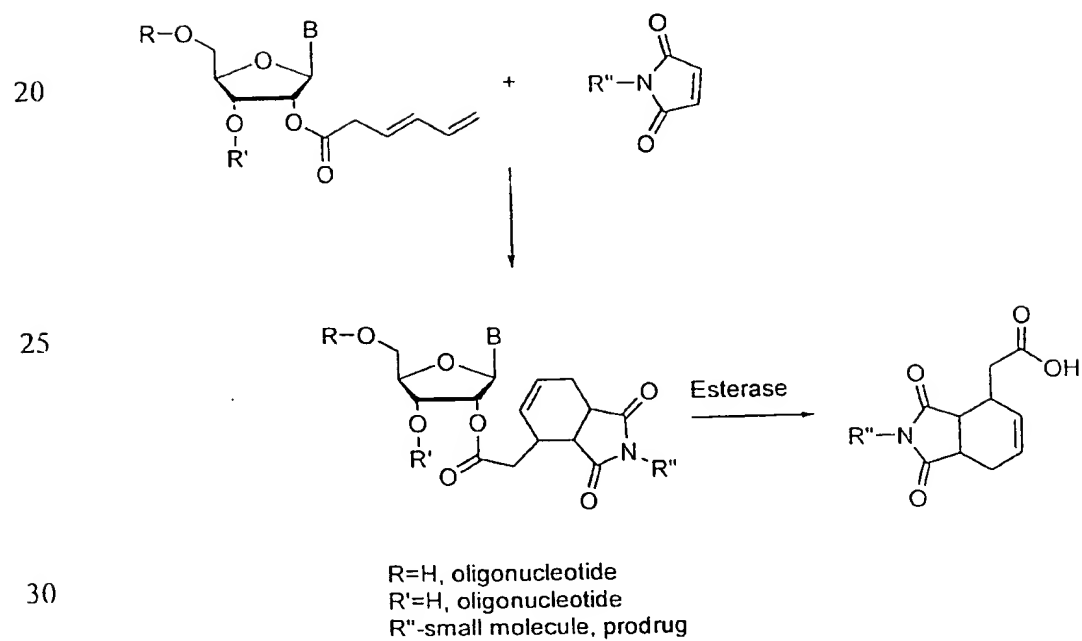
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SCHEME 27



SCHEME 28



CLAIMS

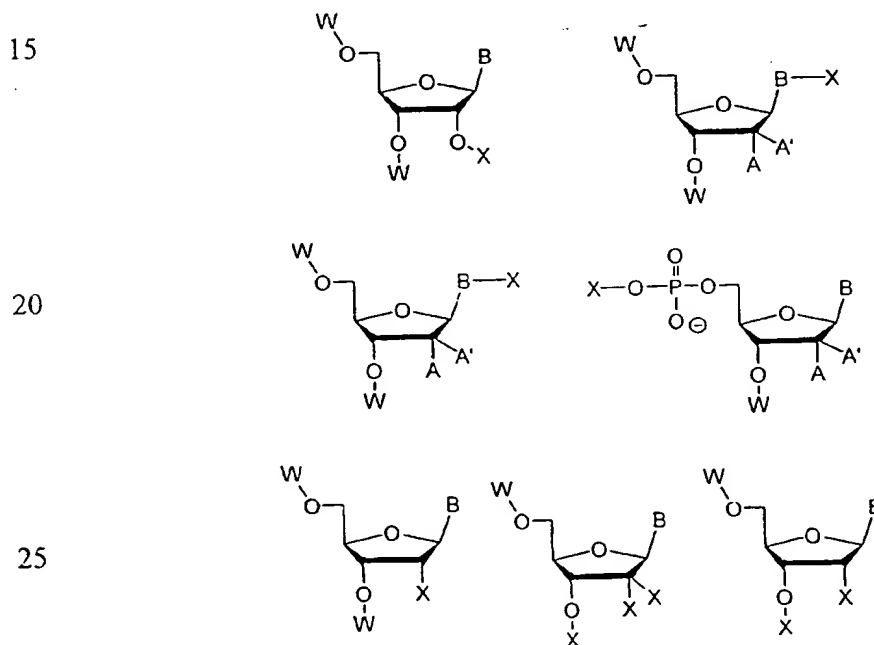
1. A method for bioconjugating macromolecules comprising the step of reacting a derivatized macromolecule with a derivatized molecular entity capable of reacting with said derivatized macromolecule via a cycloaddition reaction.
5
2. The method of claim 1 wherein said cycloaddition reaction is selected from the group consisting of a Diels-Alder reaction, a 1,3-dipolar cycloaddition, a [2+2] cycloaddition, reaction, a ketene cycloaddition and an ene cycloaddition reaction.
10
3. The method of claim 1 wherein said macromolecule is selected from the group consisting of nucleic acids, oligonucleotides, proteins, peptides, carbohydrates, polysaccharides, glycoproteins, lipids, hormones, drugs or prodrugs.
15
4. The method of claim 1 wherein said molecular entity is selected from the group consisting of a macromolecule, antibody, polymer, resin, non-immunogenic high molecular weight compound and a diagnostic detector molecule.
20
5. The method of claim 4 wherein said diagnostic detector molecule is selected from the group consisting of fluorescent, chemiluminescent, radioisotope and bioluminescent marker compounds, antibodies and biotin.
25
6. The method of claim 4 wherein said diagnostic detector molecule is selected from the group consisting of a dieneophile derivatized fluorescein, coumarin and a metal chelator.
30
7. The method of claim 6 wherein said dienophile is a maleimide.
8. The method of claim 4 wherein said polymer is selected from polyethylene glycol or polystyrene.

9. The method of claim 1 wherein said macromolecule is derivatized with a moiety selected from the group consisting of a diene, dienophile and 1,3-dipolarophile.

5 10. The method of claim 1 wherein said molecular entity is derivatized with a group selected from the group consisting of a diene, dienophile and 1,3-dipolarophile.

11. The method of claim 1 wherein said derivatized macromolecule is an
10 oligonucleotide.

12. The method of claim 11 wherein said derivatized oligonucleotide is selected from the group of compounds having the following structure:



wherein

B is a nucleobase;

30 A and A' are 2'-sugar substituents;

W is independently selected from the group consisting of an oligonucleotide having between 1-1000 nucleobases, or H; and

X is a diene, dienophile, 1,3-dipolarophile, 1,3-dipole or other moiety capable of undergoing a cycloaddition reaction additionally, when X is attached to the nucleobase B it can be attached to a carbon atom, an exocyclic nitrogen or an exocyclic oxygen.

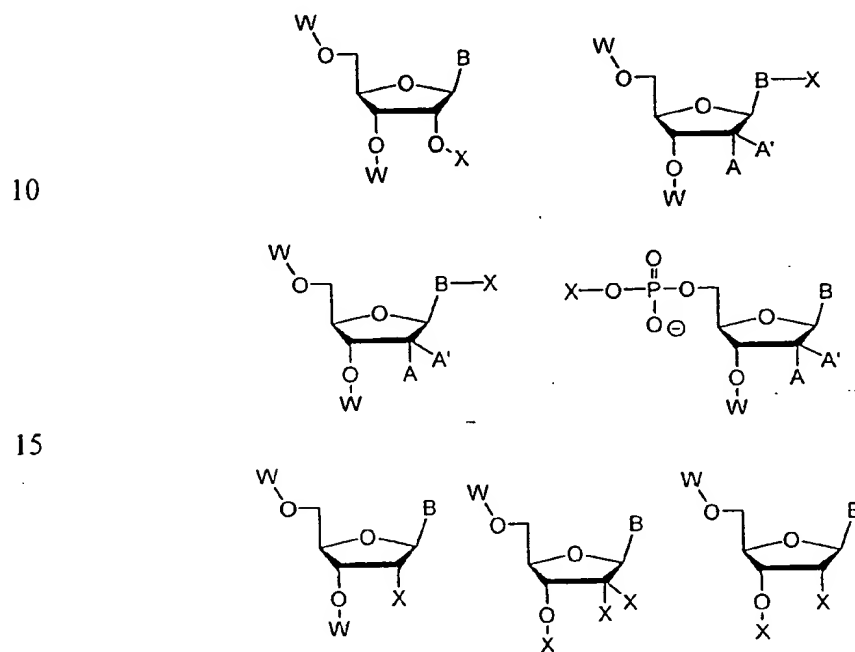
13. The method of claim 12 wherein

A and A' are independently selected from the group consisting of H, ^2H , ^3H , Cl, F, OH, NHOR^1 , NHOR^3 , NHNHR^3 , NHR^3 , $=\text{NH}$, CHCN , CHCl_2 , SH, SR^3 , CFH_2 , CF_2H , CR_2Br , $-(\text{OCH}_2\text{CH}_2)_n\text{OCH}_3$, OR^4 and imidazole;
 R^1 is selected from the group consisting of H and an alcohol protecting group;
 R^2 is selected from the group consisting of $=\text{O}$, $=\text{S}$, H, OH, CCl_3 , CF_3 , halide, optionally substituted C_1 - C_{20} alkyl (including cyclic, straight chain, and branched), alkenyl, aryl, C_1 - C_{20} acyl, benzoyl, OR^4 and esters;
 R^3 is selected from the group consisting of R^2 , R^4 , CN, $\text{C}(\text{O})\text{NH}_2$, $\text{C}(\text{S})\text{NH}_2$, $\text{C}(\text{O})\text{CF}_3$, SO_2R^4 , amino acid, peptide and mixtures thereof;
 R^4 is selected from the group consisting of an optionally substituted hydrocarbon (C_1 - C_{20} alkyl, C_2 - C_{20} alkenyl, C_2 - C_{20} alkynyl), an optionally substituted heterocycle, t-butyldimethylsilyl ether, triisopropylsilyl ether, nucleoside, carbohydrate, fluorescent label and phosphate; and
X is selected from the group consisting of an alkyl or substituted alkyl group bearing a conjugated diene unit, an alkoxy or substituted alkoxy group bearing a conjugated diene unit, $\text{CH}_2=\text{CHCH}=\text{CHCH}_2\text{CH}_2\text{O}-$, maleimide substituted alkoxy groups, dienophile substituted alkoxy groups, alkoxy groups, an alkylamino or substituted alkylamino group bearing a conjugated diene unit, maleimide substituted alkylamino groups or substituted alkylamino groups, an alkylamino group or substituted alkylamino group bearing a dienophile moiety.

14. The method of claim 13 wherein A is selected from the group consisting of H, OH, NH_2 , Cl, F, NHOR^3 , OR^4 and OSiR^4 .

15. The method of claim 1 wherein said cycloaddition reaction is a Diels-Alder reaction.

16. A derivatized oligonucleotide is selected from the group of
 5 compounds having the following structure:



wherein

B is a nucleobase;

A and A' are 2'-sugar substituents;

W is independently selected from the group consisting of an oligonucleotide having between 1-1000 nucleobases, or H; and

25 X is a diene, dienophile, 1,3-dipolarophile, 1,3-dipole or other moiety capable of undergoing a cycloaddition reaction additionally. when X is attached to the nucleobase B it can be attached to a carbon atom, an exocyclic nitrogen or an exocyclic oxygen.

30

17. The derivatized oligonucleotide of claim 16 wherein

A and A' are independently selected from the group consisting of H, ^2H , ^3H , Cl, F, OH, NHOR^1 , NHOR^3 , NHNHR^3 , NHR^3 , $=\text{NH}$, CHCN , CHCl_2 , SH, SR^3 , CFH_2 , CF_2H , CR^2_2Br , $-(\text{OCH}_2\text{CH}_2)_n\text{OCH}_3$, OR^4 and imidazole;

5 R^1 is selected from the group consisting of H and an alcohol protecting group;

R^2 is selected from the group consisting of $=\text{O}$, $=\text{S}$, H, OH, CCl_3 , CF_3 , halide, optionally substituted $\text{C}_1\text{-C}_{20}$ alkyl (including cyclic, straight chain, and branched), alkenyl, aryl, $\text{C}_1\text{-C}_{20}$ acyl, benzoyl, OR^4 and esters;

R^3 is selected from the group consisting of R^2 , R^4 , CN, $\text{C}(\text{O})\text{NH}_2$, $\text{C}(\text{S})\text{NH}_2$, $\text{C}(\text{O})\text{CF}_3$,

10 SO_2R^4 , amino acid, peptide and mixtures thereof;

R^4 is selected from the group consisting of an optionally substituted hydrocarbon ($\text{C}_1\text{-C}_{20}$ alkyl, $\text{C}_2\text{-C}_{20}$ alkenyl, $\text{C}_2\text{-C}_{20}$ alkynyl), an optionally substituted heterocycle, t-butyldimethylsilyl ether, triisopropylsilyl ether, nucleoside, carbohydrate, fluorescent label and phosphate; and

15 X is selected from the group consisting of an alkyl or substituted alkyl group bearing a conjugated diene unit, an alkoxy or substituted alkoxy group bearing a conjugated diene unit, $\text{CH}_2=\text{CHCH}=\text{CHCH}_2\text{CH}_2\text{O}-$, maleimide substituted alkoxy groups, dienophile substituted alkoxy groups, alkoxy groups, an alkylamino or substituted alkylamino group bearing a conjugated diene unit, maleimide substituted alkylamino groups or substituted alkylamino groups, an alkylamino group or substituted alkylamino group bearing a dienophile moiety.

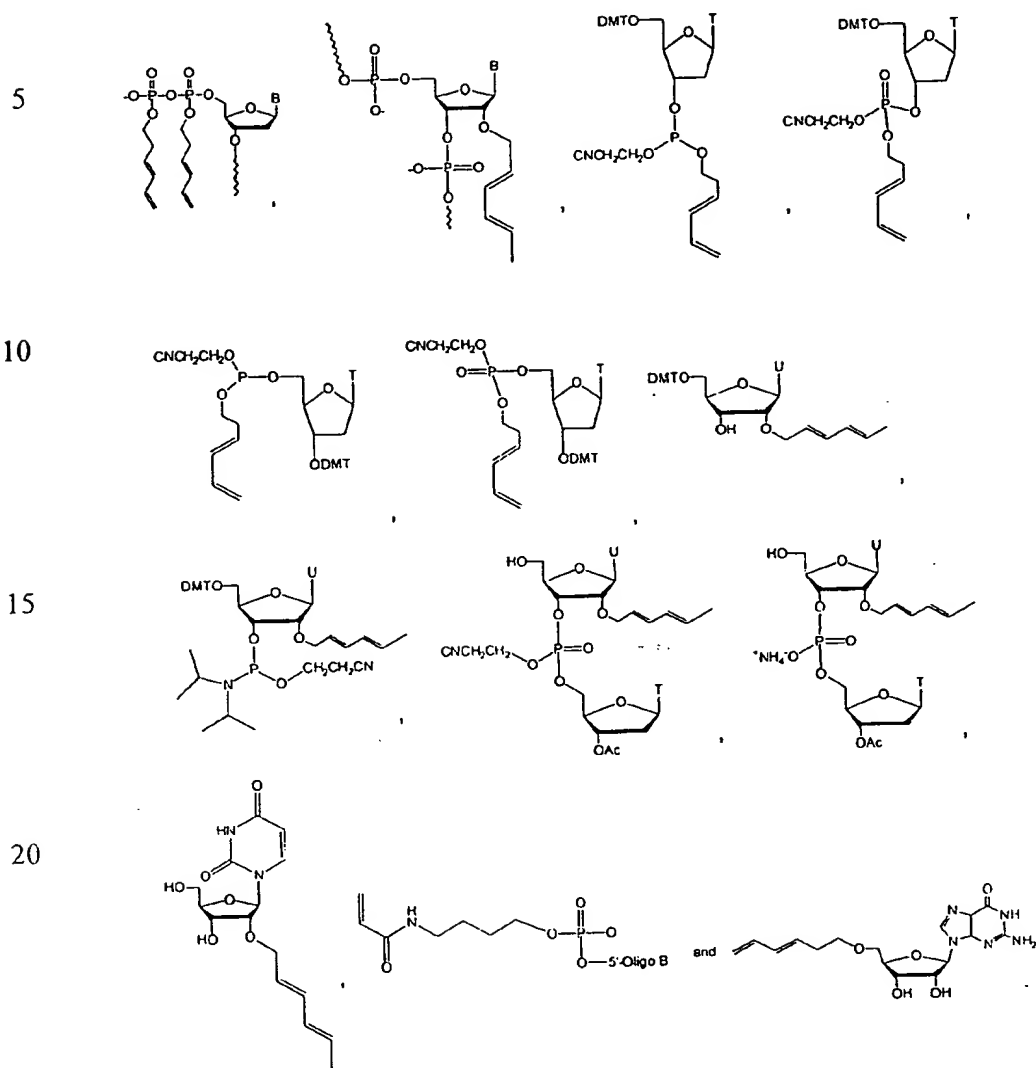
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18. The derivatized oligonucleotide of claim 17 wherein A is selected from the group consisting of H, OH, NH_2 , Cl, F, NHOR^3 , OR^4 and OSiR^4_3 .

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19. The derivatized oligonucleotide of claim 16 selected from the group consisting of



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21. The bioconjugated product of claim 20 selected from the group consisting of

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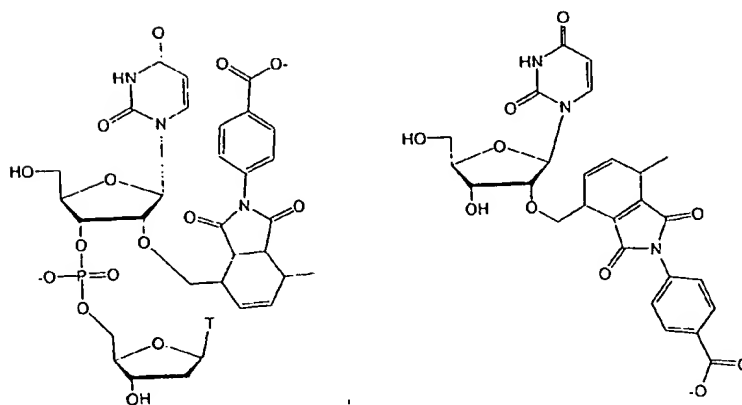
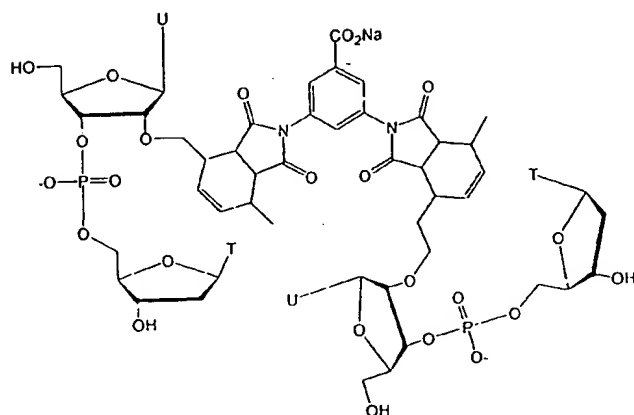
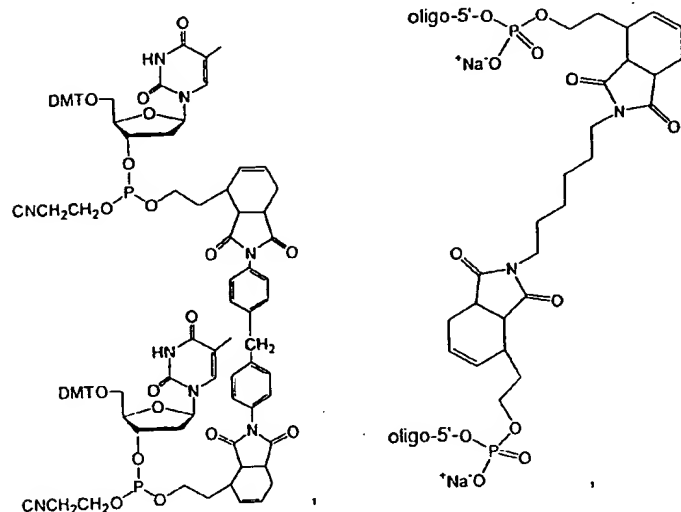
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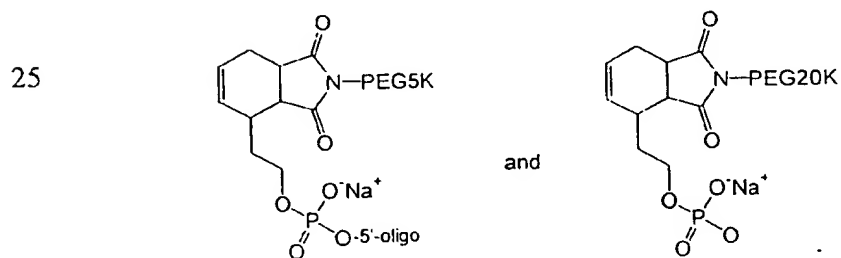
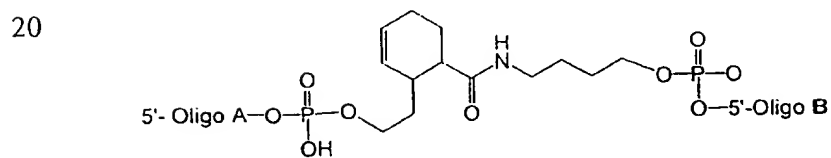
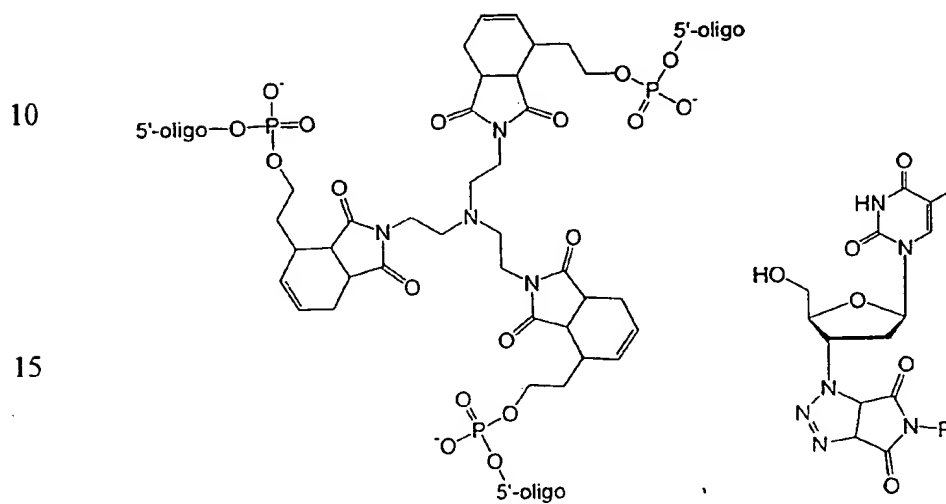
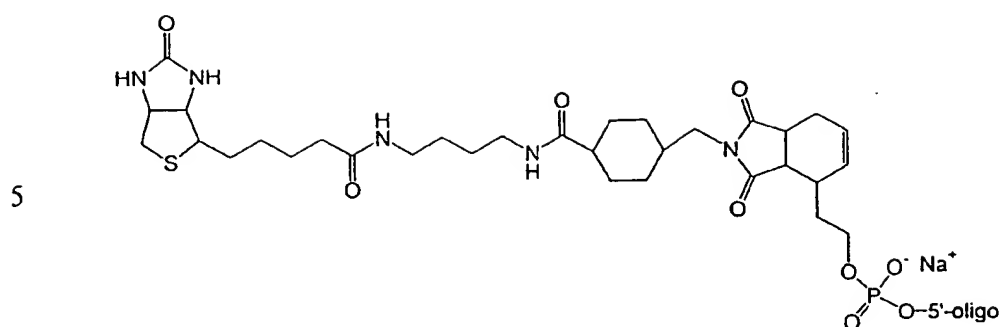
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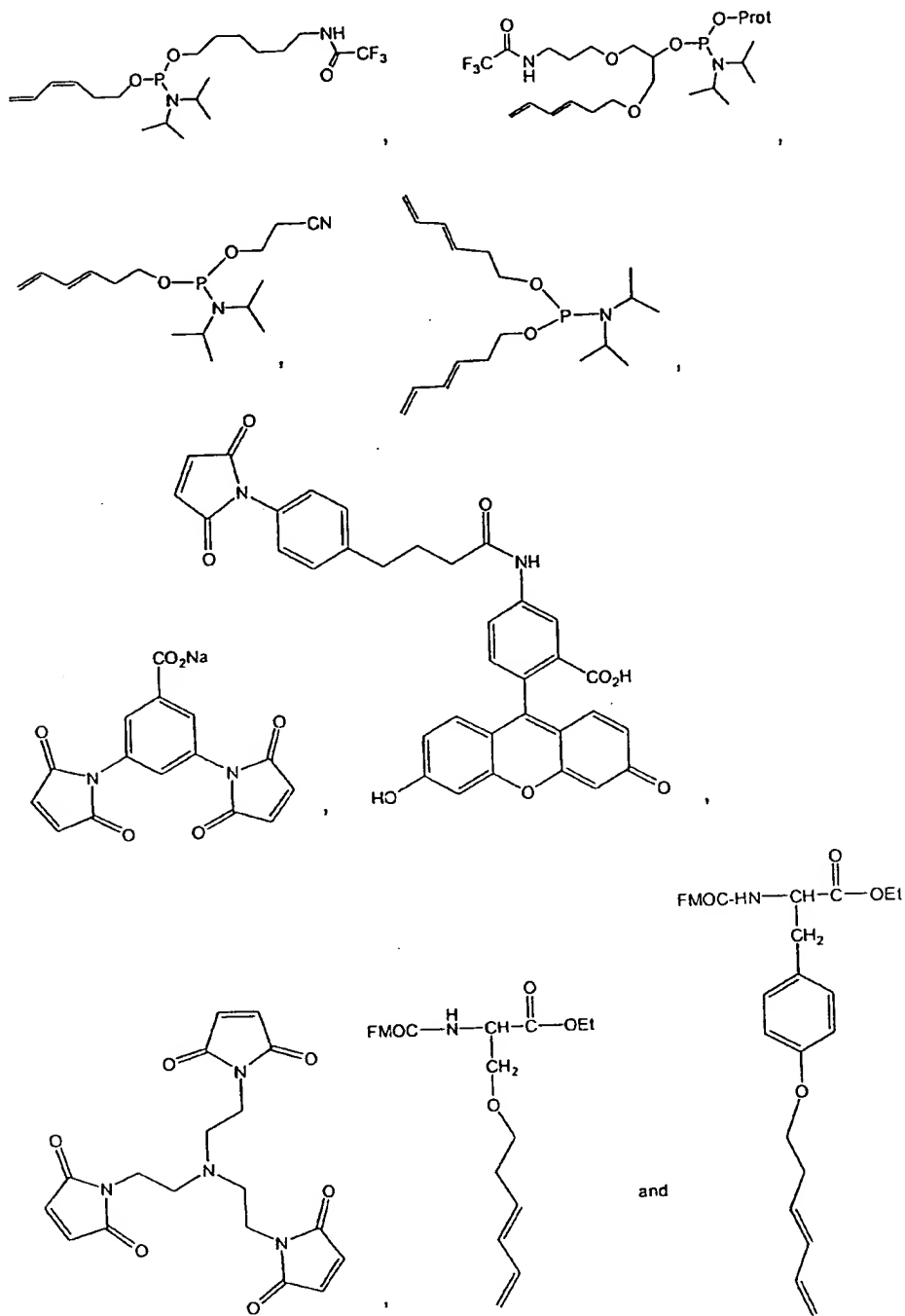


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22. A compound selected from the group consisting of



wherein Prot is an alcohol protecting group.

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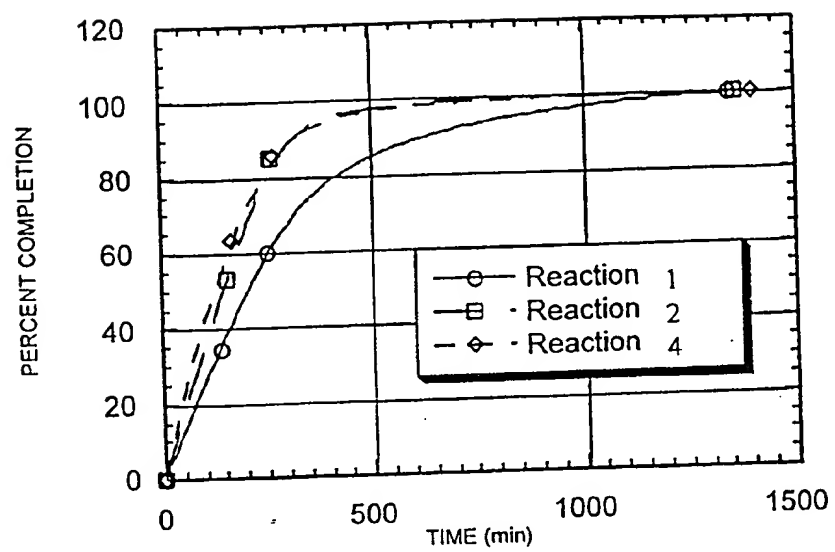


Fig. 1

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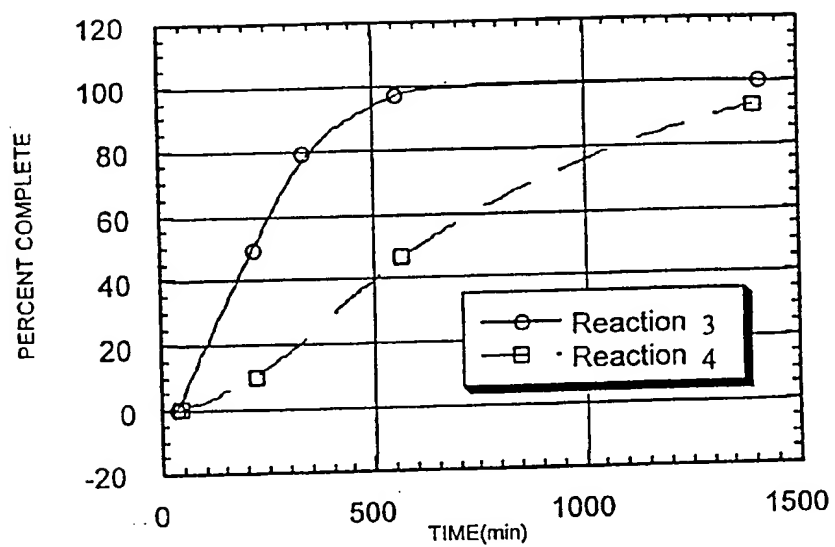


Fig. 2

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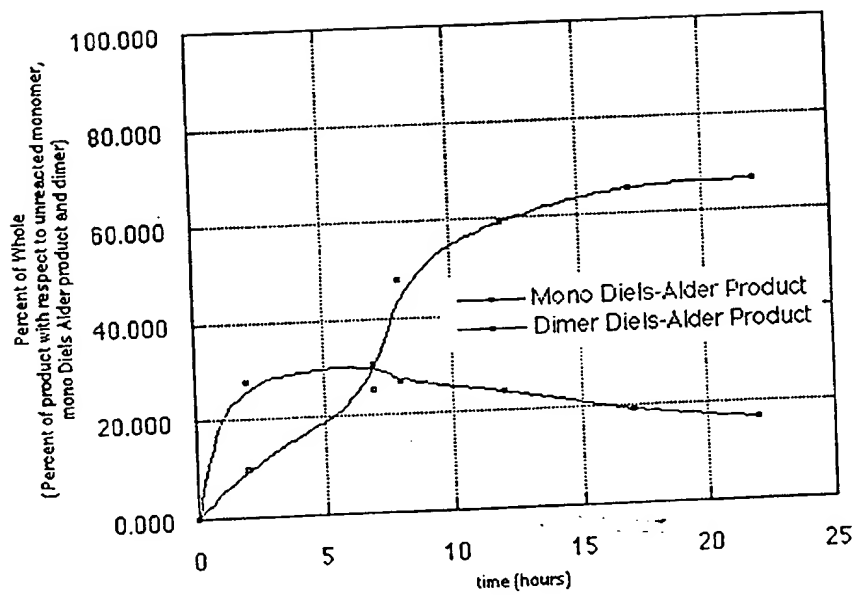


Fig. 3

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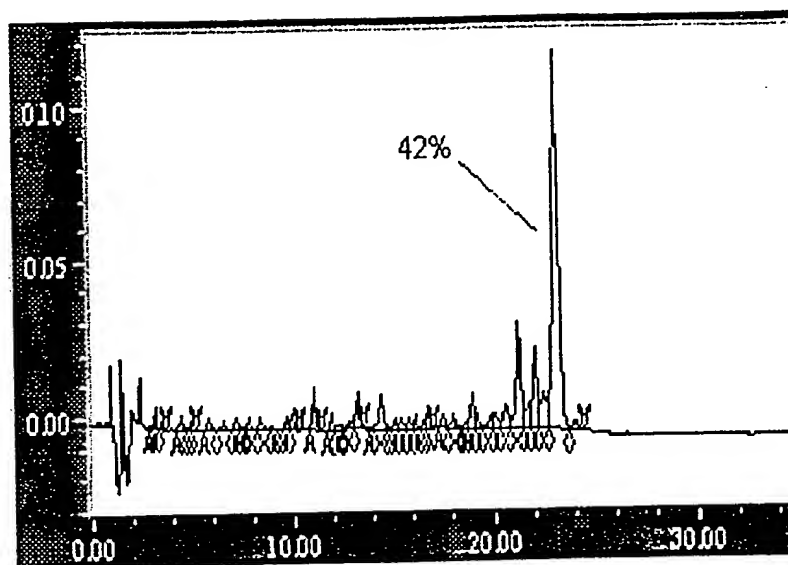


Fig. 4

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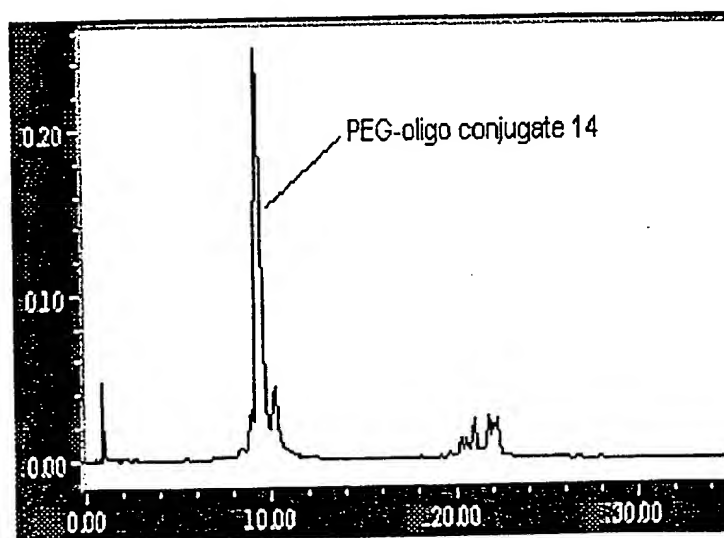


Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/00649

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-21

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/00649

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

Group I, claim(s) 1-21, drawn to a method for bioconjugating macromolecules comprising the step of reacting a derivatized macromolecule with a derivatized molecular entity via a cycloaddition reaction.

Group II, claim(s) 22, drawn to a compound wherein Prot is an alcohol protecting group.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is drawn to a method for bioconjugating macromolecule involving a cycloaddition reaction, Group II is drawn to a compound wherein Prot is an alcohol protecting group.